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CHARACTERIZATION OF THE *TRIM37* GENE AND
MUTATIONS UNDERLYING MULIBREY NANISM

Riikka Hämäläinen

Folkhälsan Institute of Genetics

Department of Medical Genetics
and
Neuroscience Center
University of Helsinki

Academic dissertation

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Supervised by

Professor Anna-Elina Lehesjoki, MD, PhD
Folkhälsan Institute of Genetics and
Neuroscience Center
University of Helsinki
Helsinki, Finland

Reviewed by

Docent Marjo Kestilä, PhD
Department of Molecular Medicine
National Public Health Institute
Helsinki, Finland

and

Docent Sirpa Kivirikko, MD, PhD
Laboratory of Molecular Genetics
Helsinki University Hospital
Helsinki, Finland

Official opponent

Professor Jaakko Ignatius, MD, PhD
Department of Clinical Genetics
University of Oulu
Oulu, Finland

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to by their Roman numerals in the text. In addition some unpublished data are presented.

- I Hämäläinen RH, Avela K, Lambert JA, Kallijärvi J, Eyaid W, Gronau J, Ignaszewski AP, McFadden D, Sorge G, Lipsanen-Nyman M, Lehesjoki AE. Novel mutations in the *TRIM37* gene in mulibrey nanism. *Hum Mutat* 23:522, 2004.

- II Hämäläinen RH, Mowat D, Gabbet MT, O'Brien TA, Kallijärvi J, Lehesjoki AE. Wilms' tumor and novel *TRIM37* mutations in an Australian patient with mulibrey nanism. *Clin Genet*, in press.

- III Hämäläinen RH, Joensuu T, Kallijärvi J, Lehesjoki AE. Characterization of the mulibrey nanism-associated *TRIM37* gene: transcription initiation, promoter region and alternative splicing. *Gene* 366:180-188, 2006.

- IV Kallijärvi J, Hämäläinen RH, Karlberg N, Sainio K, Lehesjoki AE. Tissue expression of the mulibrey nanism-associated Trim37 protein in embryonic and adult mouse tissues. *Histochem Cell Biol* 126:325-334, 2006.

Publications I and IV also appear in the thesis of Jukka Kallijärvi (2006).

ABBREVIATIONS

aa	amino acid
ATP	adenosine triphosphate
BHK	baby hamster kidney cells
BLAST	basic local alignment search tool
bp	base pair
c.	coding DNA reference sequence position
cDNA	complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humain
CNS	central nervous system
COS-1	african green monkey kidney cells
del	deletion
DNA	deoxyribonucleic acid
dup	duplication
dsRNA	double stranded RNA
E	embryonic day
EJC	exon junction complex
ES	embryonic stem cell
EST	expressed sequence tag
FMF	familial mediterranean fever
fs	frame shift
GST	glutathione-S-transferase
HA	hemagglutinin
IGT	impaired glucose tolerance
ins	insertion
IRS	insulin resistance syndrome
IUGR	intrauterine growth restriction
kb	kilobase
miRNA	micro RNA
mRNA	messenger RNA
NMD	nonsense mediated decay
nt	nucleotide
OMIM	online mendelian inheritance in man
ORF	open reading frame
OS	Opitz syndrome
p.	protein reference sequence position
PAGE	polyacrylamide gel electrophoresis
PBD	peroxisome biogenesis disorder
PCR	polymerase chain reaction
PTC	premature termination codon
RBCC	RING-B-Box-Coiled-coil
RNA	ribonucleic acid
RT-PCR	reverse transcriptase PCR
siRNA	small interfering RNA
SD	standard deviation
SGA	small for gestational age
SRS	Silver-Russell syndrome
SSCP	single strand conformational polymorphism
TNF	tumor necrosis factor
TRAF	tumor necrosis factor receptor associated factor
TRIM	tripartite motif
UTR	untranslated region

ABSTRACT

Mulibrey nanism is a developmental disorder with autosomal recessive inheritance. It is characterized by prenatal onset growth failure without postnatal catch-up growth, distinctive craniofacial features, progressive cardiopathy and failure of sexual maturation. Besides the developmental features, the patients develop insulin resistance syndrome and type 2 diabetes at an early age and they have an increased risk of developing tumors. Mulibrey nanism is a rare disorder, with less than 120 patients reported worldwide. As 80% of all diagnosed patients come from Finland, mulibrey nanism is a classical example of a disorder belonging to the Finnish disease heritage. The molecular pathogenesis underlying this disorder is unknown.

Prior to this study, the gene underlying mulibrey nanism was positionally cloned in the year 2000 and identified as a novel member of the RBCC (RING-Box-coiled-coil) protein family. In 2001 the gene was given its present name, *TRIM37*, when the tripartite motif (TRIM) protein family was characterized. In 2002, mulibrey nanism was classified as a peroxisomal disorder when the *TRIM37* protein was shown to localize to peroxisomes.

The physiological function of *TRIM37* and the pathogenetic mechanisms leading from *TRIM37* dysfunction to the mulibrey nanism phenotype remain unknown. However, while this study was ongoing *TRIM37* was shown to possess ubiquitin E3 ligase activity and thus possibly mediate ubiquitin dependent protein degradation, suggesting that accumulation of yet unknown substrate proteins may underlie the disease pathogenesis.

In this study, the *TRIM37* gene was characterized in detail. Besides characterization of the complete genomic structure of the gene, a transcription initiation window, with several separate transcription start sites, was identified and the putative promoter region immediately upstream of the transcription initiation window was shown to possess basal promoter activity. Further, several alternative splice variants of the gene were identified, including a highly expressed testis specific variant, encoding an identical protein product with the main transcript. Expression of *TRIM37* mRNA was detected in several different tissues, with highest expression seen in testis and in brain, when the expression patterns of the two major transcripts in different human tissues were studied by quantitative real-time PCR.

Several mulibrey nanism patients of different ethnic backgrounds were studied and thirteen novel mutations in *TRIM37* were found. While most mutations predict truncation of the protein products and most likely lead to mRNA degradation, three novel mutations predict translation of mutated proteins (p.Cys109Ser, p.Gly322Val and p.Glu271_Ser287del). These three mutations were further shown to result in altered subcellular localization of the mutant proteins. All the *TRIM37* mutations identified to date predict loss-of normal function alleles, and thus no genotype-phenotype correlation is seen among the patients.

In order to fully understand the pathogenetic mechanisms leading from *TRIM37* dysfunction to the mulibrey nanism phenotype, an animal model for the disorder is needed. For the development of a *Trim37* knock-out mouse, the mouse *Trim37* gene that is highly homologous to the human gene was studied in detail. The genomic organization was characterized and alternative splice variants, including a testis specific variant predicting a longer protein product, were identified. Further, the expression of *Trim37* was studied in detail in developing and adult mouse tissues and a restricted and cell specific pattern was detected by immunohistochemical methods.

This thesis work provides new tools for the molecular diagnosis of mulibrey nanism as well as for the further study of *TRIM37* function and the pathogenetic mechanisms behind this interesting disorder.

INTRODUCTION

In the early 1990's a significant number of researchers were working towards identification of the genes behind a group of disorders overrepresented in Finland. This group, comprising 36 monogenic disorders, is called the Finnish disease heritage. The definition is somewhat ambiguous and whether some diseases should be included or not is under debate. However, essential to the group is that even though the diseases are rare, a considerably higher incidence is seen in the Finnish population than in other populations, due to the population history of Finland and founder effects (Norio, 2003a). Mulibrey nanism, a growth disorder associated with abnormalities in several organs, is one of the best examples of these diseases, with nearly 90 patients known in Finland and less than 30 patients reported elsewhere in the world (Karlberg et al., 2004a).

Now more than ten years later, after countless hours of work and aided by the data of the human genome project, the genes behind most of the disorders of the Finnish disease heritage have been identified and the underlying mutations characterized (Norio, 2003b). However, the molecular mechanisms leading from the dysfunctional genes to the disease phenotypes still remain unknown in many cases. To tackle the interesting question of how a certain mutation in a certain gene can lead to the phenotype seen in patients is not easy. To identify the pathogenetic mechanisms and biological pathways behind a disorder, detailed information on the dysfunctional gene and its protein product are needed. Important information includes, for example, the normal physiological function of the protein, the biological pathways the protein is involved in, when and where it is expressed and how the expression is regulated, what other proteins interact with the protein in question and how the disruption affects them. Knowledge of the gene and protein structure and the type of mutations that underlie the disease can shed some light on these questions, and some hypotheses based on these data can be tested with cellular models. However, sometimes the only way to answer these questions is through an animal model.

The laboratory mouse, *Mus musculus domesticus*, is the principle mammalian species used as a model for human biology in biomedical research. Lower species, like worms, flies and fish, have their advantages, but the level of genetic homology and the similarities in embryogenesis make the mouse the most used model organism

for human diseases. Currently, the mouse germ line can be manipulated in almost every conceivable way through genetic modification of mouse ES cells, there are large scale mutagenesis programs that produce thousands of new mouse mutants every year, and the mouse genome sequence is available in public databases. These facts will assure that the mouse will most likely keep its position as the number one animal to model human disease also in future decades. However, mice and humans are not identical and even high homology between human and mouse orthologs does not ensure that the mouse model will replicate the human phenotype.

To successfully generate a mouse model by targeted disruption of a specific gene, detailed information of the gene of interest should be available. Important questions to be answered include: will the disruption of the gene in question disrupt other genes or regulatory regions affecting other genes, are there alternative splice variants that may still produce functional proteins and is the gene expressed in the same tissues in mouse and man. Frequently a mouse model is designed to mimic a human mutation, however, this does not necessarily overcome the possible problems mentioned above.

Apart from the characterization of the human *TRIM37* gene and the mutations underlying mulibrey nanism, one aim of this study was to establish the foundation for the generation of *Trim37* knock-out mice. Characterization of the mouse *Trim37* gene and the distribution of Trim37 expression in developing and adult mouse tissues was the first step in this process, which is currently ongoing. The first homozygous Trim37 knock-out mice have just been born and in the future they will hopefully help to elucidate the pathogenetic mechanisms behind mulibrey nanism.

REVIEW OF THE LITERATURE

1. Growth retardation

Variation in human height is fairly large and even seemingly unusual growth patterns can be regarded as within the normal range. For example, in familial short stature the children are short merely because their parents are short, and temporarily slow growth with catch-up growth compensating for final height is typical for late puberty (Rosenthal and Wilson, 1994).

Abnormal short stature is considered when a persons height is 2.5 standard deviation (SD) below the mean height for age and gender or for midparental height (Rosenthal and Wilson, 1994). Growth is dependent on several different factors and whether pre- or postnatal, abnormal short stature can be due to either primary i.e. intrinsic factors that work from inside the cells, or secondary factors i.e. extrinsic factors that originate from the environment.

1.1 Prenatal growth retardation

The World Health Organization defines 'low birth weight' as below 2500 g. About 15% of all infants are born with low birth weight (Unicef statistics 2005), and the level is twice as high in developing countries as it is in developed regions. However, this term is irrespective of gestational age and includes babies born prematurely. Small for gestational age (SGA), on the other hand, takes into account the gestational age and describes infants with birth size below a certain centile, normally the 10th, meaning that at birth 10% of the population at that gestational age are smaller while 90% are bigger. Intrauterine growth restriction (IUGR) is defined as the failure of the infant to achieve his or her growth potential *in utero*. Thus, in IUGR the failure to grow along the consistent centile is more important than the absolute size of the infant (Ergaz et al., 2005).

Prenatal growth is a complex, dynamic process, tightly linked to cell proliferation, differentiation and organization of different organ systems. These events are controlled by a variety of genetic and environmental factors that can be divided according to maternal, placental or fetal origin. Maternal factors leading to prenatal growth restriction include exposure to toxic substances (for example smoking,

alcohol, drugs), chronic diseases, malnutrition, birth order, multiple births and maternal genetic factors. Placental factors affecting fetal growth include mainly placental insufficiency, which can be the result of vascular damage, pre-eclampsia, placental infarction, partial placental separation or simply a small placenta. Genetic and chromosomal aberrations, as well as intrauterine infection, are fetal factors behind IUGR (Rosenthal and Wilson, 1994; Ergaz et al., 2005).

IUGR can be either symmetric or asymmetric. Head-to-abdominal circumference ratios can be used to differentiate infants into proportionally small i.e. symmetrical, or those with relative head sparing (asymmetrical). Symmetrical IUGR is often due to fetal causes for example viral infections or developmental abnormalities, in which case all the organs show equal growth restriction. Asymmetrical IUGR, on the other hand, is usually due to extrinsic factors for example placental insufficiency or teratogenic substances. When suffering from malnutrition, the fetus tries to prioritize organs and maintain normal growth in the brain, while other organs are left behind (Cunningham et al., 1997).

IUGR infants show 12 times higher perinatal mortality than do infants with normal birth weight (Cunningham et al., 1997). Later in life, even after postnatal catch-up growth, they are still at greater risk of developing coronary heart disease, hypertension and type 2 diabetes (Reece and Hagay, 1999; Barker et al., 2005).

1.2 Postnatal growth retardation

Failure to thrive is a condition where an infant grows more slowly than expected. In most cases, the underlying problem is inadequate nutrition, but it may also be a symptom of another problem such as a viral infection or digestive problem. Chronic diseases, endocrine abnormalities, for example growth hormone deficiency or hypothyroidism, and genetic disorders such as Turner syndrome are other causes for postnatal growth retardation (Rosenthal and Wilson, 1994; Cowell, 1995; Ergaz et al., 2005).

1.3 Congenital growth disorders

A strong association between chromosome aberrations, IUGR and congenital malformations exists (Monk and Moore, 2004). For example, fetuses with trisomies

are frequently growth restricted, and many chromosome abnormalities include suboptimal growth of the fetus as one of the major hallmarks. Some studies have reported abnormal fetal karyotype to be responsible for one in five of all IUGR fetuses (Snijders et al., 1993).

Most of the congenital growth disorders are due to abnormal growth and remodeling of bone and/or cartilage (Baitner et al., 2000). These disorders, called chondro- or osteochondrodysplasias, are often hereditary and mutations for example in fibroblast growth factor receptors, collagens, different hormone receptors and sulfate transporters have been shown to underlie them (Baitner et al., 2000). The major clinical sign in these disorders is short stature with disproportionately short extremities, but many patients also manifest other features that often require medical attention (Baitner et al., 2000). Achondroplasia (OMIM# 100800), which is caused by mutations in *FGFR3* (Fibroblast Growth Factor Receptor 3), is the most common human chondrodysplasia (Shiang et al., 1994). Diastrophic dysplasia (OMIM# 222600) and Cartilage-hair hypoplasia (OMIM# 250250) are congenital bone and cartilage disorders enriched in the Finnish population and represent disorders of the Finnish disease heritage (Norio, 2003b).

Several other monogenic disorders manifest proportional short stature as a major landmark of the disorder. For example, Silver-Russell syndrome (SRS, OMIM# 180860) shows severe pre- and postnatal growth retardation. Other clinical features of SRS include asymmetry of the limbs and craniofacial disproportion (Silver et al., 1953; Russell, 1954). The majority of SRS cases are sporadic but some familial cases have been reported (Duncan et al., 1990; Ounap et al., 2004). Further, approximately 10% of all SRS cases are associated with maternal uniparental disomy of chromosome 7 (Hannula et al., 2001; Monk et al., 2002).

Another disorder with similar phenotype is the 3-M syndrome (OMIM# 273750). 3-M syndrome is characterized by low birth weight, proportional dwarfism, dysmorphic facial features and radiological abnormalities (Miller et al., 1975). Interestingly, the gene behind 3-M syndrome was recently identified as *cullin-7*, encoding CUL7 that assembles an E3 ubiquitin ligase complex and thus promotes ubiquitination (Huber et al., 2005). *Cul7*^{-/-} mice die immediately after birth due to respiratory distress, and show progressive growth retardation during their embryonic period. Abnormal vascular morphogenesis is seen in the *Cul7*^{-/-} placentas and dermal

and hypodermal hemorrhage is detected in mutant embryos, suggesting vascular morphogenesis as one of the underlying causes for the growth problem in 3-M syndrome (Arai et al., 2003). Together with the results that *TRIM37* underlying mulibrey nanism (see section 2) encodes a ubiquitin E3 ligase (Avela et al., 2000; Kallijärvi et al., 2005), these data suggest that impaired ubiquitination may play a role in intrauterine growth restriction in humans.

2. Mulibrey nanism

Mulibrey nanism (Muscle-Liver-Brain-Eye nanism; OMIM# 253250) is a rare prenatal onset growth disorder with autosomal recessive inheritance. It was first described in Finland in the 1970's by Jaakko Perheentupa (Perheentupa et al., 1970; Perheentupa et al., 1973), and has also been known as Perheentupa syndrome. To date there are approximately 120 patients diagnosed with mulibrey nanism worldwide, with the majority of them (~90) from Finland. However, sporadic patients with mulibrey nanism have been reported from various ethnic groups around the world (Thoren, 1973; Cumming et al., 1976; Voorhess et al., 1976; Similä et al., 1980; Sanchez-Corona et al., 1983; Cotton et al., 1988; Haraldsson et al., 1993; Seemanova and Bartsch, 1999; Avela et al., 2000; Jagiello et al., 2003).

The clinical picture of a mulibrey nanism patient can be variable. Due to this, and the overlap of the phenotype with other growth disorders, the diagnosis is sometimes difficult. Overlapping phenotypic features with mulibrey nanism are seen for example in Silver-Russell and 3-M syndromes (see previous section 1.3).

2.1 Mulibrey nanism – a member of the Finnish disease heritage

The Finnish disease heritage is a group of rare monogenic diseases comprising 36 different disorders that are more common in Finland than elsewhere in the world (Norio, 2003a). The reason why some hereditary diseases are overrepresented in Finland, while some that are relatively common in other populations are extremely rare or absent in the Finnish population, lies in the population history of Finland (Norio et al., 1973; de la Chapelle, 1993). A small and restricted number of founders, national and regional isolation, as well as several population bottlenecks have lead to enrichment of some disease alleles while others have been eliminated from the genomes of Finns. Thus, these disorders all have one or two founder mutations enriched in the isolated Finnish population that account for the majority of the cases inside Finland (Norio, 2003a).

Mulibrey nanism is a perfect example of a disease belonging to the Finnish disease heritage. It is clearly more common in Finland than elsewhere, most of the Finnish patients come from clustered regions in Savo and North Carelia (Lipsanen-

Nyman, 1986; de la Chapelle, 1993), and in the Finnish patients there is one major mutation accounting for 98% of all Finnish disease chromosomes (Avela et al., 2000).

2.2 Clinical characteristics of mulibrey nanism

The most characteristic feature of mulibrey nanism is prenatal onset growth restriction, without postnatal catch-up growth. The mean height is -3.1 SD at birth and -4.0 SD at the age of diagnosis, and the adult height of mulibrey patients is on average 33 cm below the mean adult height in the population (Karlberg et al., 2004a). Further, the patients are gracile and have thin extremities. Over 90% of the patients show typical craniofacial features including triangular face with prominent forehead, low nasal bridge and scaphocephaly (Figure 1). Other typical findings include J-shaped sella turcica, peculiar high voice, hepatomegaly, yellowish dots in the ocular fundi, cutaneous naevi flammei, and fibrous dysplasia of long bones (Karlberg et al., 2004a).

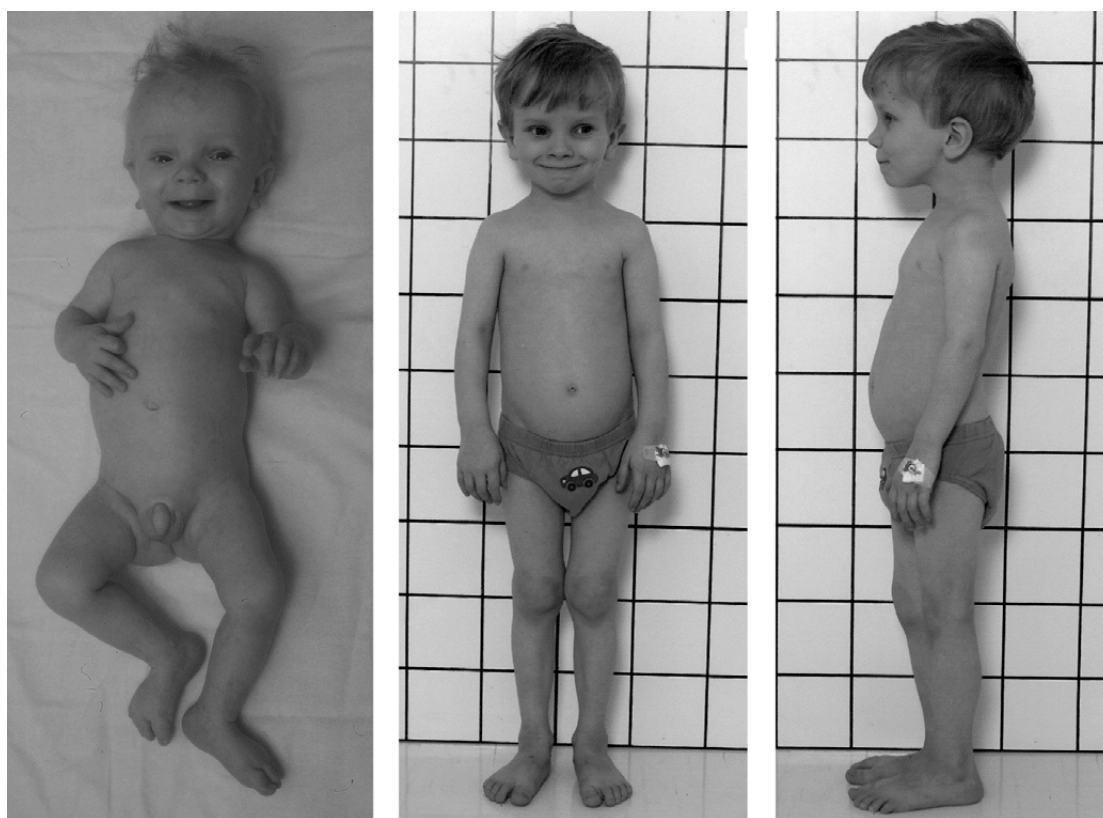


Figure 1. A boy with mulibrey nanism at the age of 8 months and 5 years.

The typical craniofacial features; triangular face, frontal bossing, low nasal bridge and the low-set ears are evident at the age of 8 months. At the age of 5 years they are still seen, but are not as striking as before. The pictures are published with informed consent from the patient and his parents.

The most severe and sometimes life threatening symptoms are due to the heart disease, constituting pericardial constriction and myocardial hypertrophy (Lipsanen-Nyman et al., 2003; Kivistö et al., 2004). Even though the patients show enlarged brain ventricles, they manifest no signs of intellectual incapacity, and the only neurological abnormality is mild muscular hypotonicity and mild delay in motor and speech development (Karlberg et al., 2004a). Hypoplasia of various endocrine glands causing hormonal deficiency and hypogonadism, as well as premature ovarian failure with subfertility, have been reported in the patients (Lipsanen-Nyman, 1986; Karlberg et al., 2004b). The main clinical features of the Finnish mulibrey nanism patients are listed in Table 1.

Table 1. Summary of the features in 85 Finnish mulibrey nanism patients at the time of diagnosis. (Karlberg et al., 2004a; Karlberg et al., 2004b; Karlberg et al., 2005)

Feature	Frequency (%)
Thin extremities	99
Short stature	96
Small, high voice	96
General gracility	95
Characteristic facial features	90
J-shaped sella turcica	89
Scaphocephaly	81
Hypoplastic tongue	80
Yellowish dots in the ocular fundi	79
Muscular hypotonicity	68
Cutaneous naevi flammei	65
Feeding difficulties during infancy	50
Hepatomegaly	45
Fibrous dysplasia of long bones	25
Renal anomaly	18
Skeletal asymmetry	15
Congestive heart failure	12
Pericardial constriction	6
Metabolic syndrome (in adults):	70
- fatty liver	100
- hypertension	81
- type 2 diabetes	50
- impaired glucose tolerance (IGT)	42
- high fasting insulin values	90
- overweight	42
Tumors:	
- fibrothecomas (in adults)	55
- liver tumors	7
- Wilms' tumor	4

The clinical diagnosis of mulibrey nanism can be challenging since growth restricted infants are a very heterogeneous group and the clinical features of mulibrey nanism are not unique but are also seen in other diseases. The diagnostic criteria of mulibrey nanism summarized by Karlberg and co-workers (Karlberg et al., 2004a) are shown in Table 2.

Table 2. Diagnostic criteria of mulibrey nanism.

For the diagnosis three major signs with one minor or two major signs with three minor are required. Adapted from Karlberg et al., 2004a.

Diagnostic sign

Major signs

Growth restriction (A, B or C)

A) SGA lacking catch-up growth

B) Childhood height 2.5 SDS below mean

C) Adult height 3.0 SDS below mean

Characteristic radiological findings (A or B)

A) slender long bones with thick cortex and narrow medullar channels

B) J-shaped sella turcica

Characteristic craniofacial features

Scaphocephaly, triangular face, high and broad forehead, low nasal bridge and telecanthus

Characteristic ocular findings

Yellowish dots in retinal midperipheral region

Mulibrey nanism in a sibling

Minor signs

Peculiar high voice

Hepatomegaly

Cutaneous naevi flammei

Fibrous dysplasia of long bones

Mulibrey nanism patients have an increased risk of developing tumors. Liver tumors have been observed in six of 85 Finnish patients (Karlberg et al., 2004a) and twelve of 22 postpubertal Finnish female patients have been diagnosed with benign ovarian tumors i.e. fibrothecomas (Karlberg et al., 2004b). Association of mulibrey nanism with childhood kidney tumors (i.e. Wilms' tumor) has also been reported, as Wilms' tumor has earlier been seen in three Finnish and one Czech patient (Seemanova and Bartsch, 1999; Karlberg et al., 2004a). Additionally, three patients included in this study have had Wilms' tumor. Interestingly, several genetic conditions that manifest excessive growth, for example Beckwith-Wiedeman syndrome (OMIM #130650), also have an increased risk for Wilms' tumor (Sotelo-Avila et al., 1980).

2.3 Metabolic findings in mulibrey nanism

Mulibrey nanism patients develop severe insulin resistance and metabolic syndrome at a very young age. While in childhood the patients show normal glucose and insulin levels, over 90% of the adults show either type 2 diabetes (50%) or impaired glucose tolerance (IGT; 42%), and the symptoms become evident at young adult life (Karlberg et al., 2005). Nearly half of the adult patients are obese and fatty liver is seen in all adult patients and in nearly half of the prepubertal children. Further, hypertension has been observed in 81% of the adults and 70% of the adult patients fulfill the criteria for the metabolic syndrome (Karlberg et al., 2005).

Metabolic syndrome is a collection of metabolic risk factors present in one person. These include abdominal obesity, atherogenic dyslipidemia, hypertension and insulin resistance or glucose intolerance (Hansen, 1999). Metabolic syndrome poses a major public health problem in western countries, since it leads to increased risks for coronary heart disease, vascular disease, stroke and diabetes (Zimmet et al., 2005). One of the underlying processes is insulin resistance. Insulin, a hormone secreted by the β -cells of the pancreas, enables cells to utilize glucose. Insulin resistance is a condition where cells do not respond to the normal amount of insulin and thus the pancreas produces additional insulin, resulting in high insulin levels. When cells do not respond even to high levels of insulin, the result is type 2 diabetes (Fletcher and Lamendola, 2004; Ten and Maclaren, 2004).

A risk for insulin resistance syndrome (IRS) has been associated with SGA (Veening et al., 2003). SGA is often due to an unfavorable environment during the fetal period causing the fetus to adapt to poor nutrition. After birth, with ample supply of nutrients, most SGA children show extensive postnatal weight gain and the risk for IRS correlates with this weight gain (McMillen and Robinson, 2005). In mulibrey nanism patients the growth retardation is not due to the fetal environment, the patients do not manifest extensive prepubertal weight gain, and the postpubertal weight gain they do manifest does not correlate with the IRS (Karlberg et al., 2005). On the other hand, the early development of fatty liver and elevated serum leptin levels suggest that the accumulation of fat in the liver may be critical for the development of IRS and metabolic syndrome in mulibrey nanism (Karlberg et al., 2005).

Overall, the metabolic phenotype of the patients makes mulibrey nanism an excellent monogenic model for type 2 diabetes, insulin resistance and metabolic syndrome (Karlberg et al., 2005).

2.4 *TRIM37* mutations – the primary defect in mulibrey nanism

A positional cloning strategy was initiated in the early 1990s to identify the causative gene for mulibrey nanism. As in many other disorders of the Finnish disease heritage, this strategy turned out to be successful also for mulibrey nanism, and in the year 2000 the underlying gene was identified when four different mutations in mulibrey nanism patients were identified in a cDNA sequence (KIAA0898; 4111 bp, predicting a 964 aa protein) coding for a novel member of the RING-B-Box-Coiled-Coil (RBCC) protein family (Avela et al., 2000). At that time the gene was named *MUL* referring to mulibrey nanism, but it was later renamed *TRIM37*, when the TRIM protein family was characterized and the TRIM37 protein was included in this family (Reymond et al., 2001).

Prior to this study, five mutations in *TRIM37* had been described in mulibrey nanism patients (Avela et al., 2000; Jagiello et al., 2003). They all produce premature termination codons (PTCs) and thus predict truncation of the protein product. The major Finnish mutation, present in 98% of all Finnish disease chromosomes, is an A to G transition on the 3' splice acceptor sequence of intron 6 (c.493-2A>G; Avela et al., 2000). This results in activation of a cryptic splice site five nucleotides downstream of the actual splice site and deletion of five nucleotides from the resulting mRNA transcript. The carrier frequency of this mutation is relatively high in the high-risk region of Finland (Savo and North-Carelia), where nine of 217 control persons (1:24) were shown to carry the disease allele (Avela et al., 2000). The other Finnish mutation, a 1-bp deletion (c.2212delG), was seen in two patients that are compound heterozygotes for this and the Finnish major mutation (Avela et al., 2000). No carriers of this mutation were detected in 316 control individuals (Avela et al., 2000).

Apart from the two Finnish mutations, three mutations in *TRIM37* had been identified in patients of non-Finnish origin. A Czech patient showed a homozygous 5-bp deletion (c.838_842delACTTT), and a homozygous 1-bp insertion (c.1346dupA) was seen in an American patient (Avela et al., 2000). A splice-site mutation affecting the 3' splice acceptor site of intron 9 (c.810-1G>A), leading to deletion of 8

nucleotides from the mRNA transcript, was reported in a Turkish family (Jagiello et al., 2003). Interestingly, phenotypic heterogeneity is seen in this family with three affected members. While one of them presents the typical clinical findings of mulibrey nanism, her two siblings are more severely affected and have additionally mental retardation (Jagiello et al., 2003). However, since all three siblings share the same mutation and mental retardation has not been reported in any other mulibrey nanism patients, it is likely that other genetic factors play a role in the mental retardation seen in this inbred family.

All five mutations lead to premature termination codons, and predict truncation of the protein products. The lengths of the putative truncated polypeptides vary significantly, with the Finnish major mutation predicting a 175 amino acid (aa) protein, with only the RING and B-Box motifs present, and the other Finnish mutation predicting a 769 aa protein with all the functional domains present in the truncated polypeptide (Avela et al., 2000). However, since PTCs induce mRNA decay through nonsense surveillance and NMD (Losson and Lacroute, 1979), truncated proteins are rarely produced and instead the truncating mutations behave as loss-of-function alleles by accelerating mRNA degradation and reducing mRNA quantity (Culbertson, 1999; Holbrook et al., 2004). This is most likely also true for the TRIM37 mutations with PTCs.

While this study was ongoing a third Finnish mutation, c.227T>C, in TRIM37 was identified in a mulibrey nanism patient (Kallijärvi et al., 2005). This mutation, observed in compound heterozygote form with the Finnish major mutation, leads to substitution of leucine by proline at aa position 76 (p.Leu76Pro) between the RING and B-box motifs of the TRIM domain. The mutation was further shown to affect the ubiquitin ligase activity of the mutated protein (Kallijärvi et al., 2005).

According to Northern blot analysis, *TRIM37* mRNA is widely expressed in adult human tissues (Avela et al., 2000). In RNA *in situ* hybridization studies expression of *TRIM37* is detected in multiple tissues during human and mouse embryogenesis. A subset of neural crest derived tissues, including dorsal root and trigeminal ganglia, show intense expression of *TRIM37*, and high expression is seen also in liver and in epithelia of ectodermal or endodermal origin of several organs whose development is regulated by mesenchymal-epithelial interactions (Lehesjoki et al., 2001).

3. The TRIM protein family

The Tripartite Motif family of proteins (TRIM), is characterized by the presence of a RING finger, one or two zinc-chelating motifs named B-boxes and a coiled-coil region (Reymond et al., 2001). Thus, it was previously named the Ring-B-Box-Coiled-Coil (RBCC) family of proteins. The order and spacing between the domains in the tripartite motif are conserved (Figure 2), suggesting that the tripartite motif is an integrated functional structure rather than a collection of separate domains (Meroni and Diez-Roux, 2005). While a RING finger is present in a variety of proteins, the B-box is the critical determinant of a tripartite motif. A few members of the TRIM protein family do not have RING domains but are still considered as members of the family because the other motifs follow the conserved order and spacing of TRIM proteins (Meroni and Diez-Roux, 2005). The TRIM domain is invariably located in the N-terminus, while various additional domains may be present in the C-terminus of TRIM proteins (Figure 2).

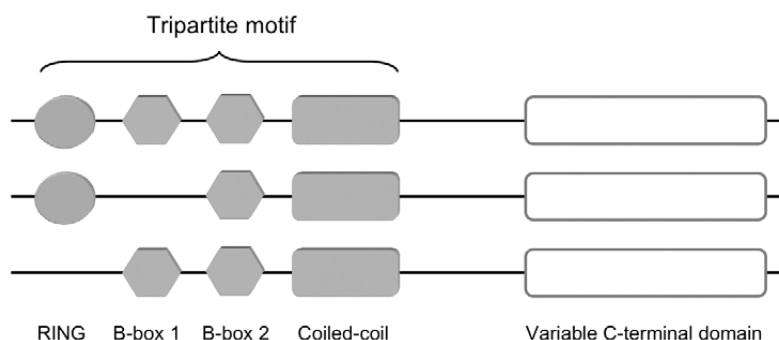


Figure 2. Schematic presentation of the domain structure of the TRIM family members.

The RING domain is defined by ordered arrangement of cysteine (C) and histidine (H) residues that bind two zinc atoms (Freemont, 1993). The other zinc-binding motif, the B-box, occurs as two types; B-box 1 and B-box 2. They share a similar but distinct pattern of C and H residues (Borden et al., 1993; Reymond et al., 2001). If both are present in a TRIM protein, the type 1 B-box always precedes the type 2 B-box, and if only one B-box is present it is always type 2 (Reymond et al., 2001). The cysteine-rich zinc binding motifs of the TRIM domain, the RING and the B-box, are believed to mediate protein-protein interactions (Borden, 1998). The coiled-coil domain that follows the type 2 B-box is also involved in interactions, promoting

formation of large protein complexes and possibly being the domain responsible for multimerization of TRIM proteins (Reymond et al., 2001). The primary sequence of the coiled-coil region is not conserved, only the hydrophobic aas (often leucines) that are required for the distinctive folding of the motif, i.e. coiling, are present at conserved positions (Lupas, 1996).

3.1 The RING finger family of E3s

The RING finger is a cysteine-rich zinc-binding sequence motif with conserved spacing of the zinc ligands (Freemont, 1993). RING fingers are defined by a consensus sequence, $CX_2CX_{9-39}CX_{1-3}HX_{2-3}C/HX_2CX_{4-48}CX_2C$, and they can be classified into two categories, RING-HC and RING-H2, depending whether the fifth zinc-binding residue is cysteine (-HC) or histidine (-H2) (Joazeiro and Weissman, 2000). Initially, the RING finger motif was thought to function only in the dimerization of proteins, but several reports have shown that it plays a critical role in mediating ubiquitin transfer from the ubiquitin conjugating enzyme to its substrates, and thus acts as a ubiquitin E3 ligase. How this is accomplished is unknown. Regulation of the E3 ligase activity of the RING finger proteins is achieved through phosphorylation or interaction with other proteins (Joazeiro and Weissman, 2000).

There are two different types of RING finger E3s. A single-subunit RING E3 consists of solely the RING finger protein, while in a multisubunit RING E3 the RING finger is part of a functional multiprotein complex (Pickart, 2001a). The RING finger E3s regulate a diverse set of cellular functions. They have a role in the cell division cycle (Joazeiro and Weissman, 2000), they have been implicated in tumorigenesis and metastasis (Fang et al., 2003) and they can contribute to apoptosis (Yang et al., 2000).

Despite the great variety of cellular processes in which the TRIM proteins are involved, the conserved integrated modular structure suggests a general function for the TRIM domain and thus a common biochemical function for all TRIM proteins. As the RING domain is strongly associated with ubiquitin E3 ligase activity and E3 activity has also been reported for some TRIM proteins (Joazeiro and Weissman, 2000), it has been proposed that TRIM proteins may represent a novel class of single protein RING finger ubiquitin E3 ligases (Meroni and Diez-Roux, 2005).

3.1.1 Ubiquitin dependent protein degradation

In order for a cell to survive, it has to be able to quickly recognize and remove misfolded or otherwise abnormal proteins. For example, regulation of the cell cycle is highly dependent on protein degradation (Glutzer, 1995). One mechanism to mark proteins for degradation is ubiquitination.

Ubiquitin, a small 76 aa protein, is highly conserved. Human, mouse and *Drosophila* ubiquitins show 100% homology in their aa sequence, and even yeast ubiquitin shows 96% homology to mammalian ubiquitin. The high evolutionary conservation implies an essential role for ubiquitin in all organisms, and yeast studies have proven ubiquitin essential for cell viability (Finley et al., 1994). All known functions of ubiquitin are mediated through its conjugation to other proteins, where it serves as a signaling molecule. Interpretation of the ubiquitin signal depends on the topology of the linkage and the number of linked ubiquitin molecules. Monoubiquitination serves as an intracellular sorting signal for internalizing membrane proteins or targeting proteins for endocytosis (Hicke, 1999; Hicke and Dunn, 2003), while a polymer of ubiquitins targets proteins for proteasomal degradation (Hershko and Ciechanover, 1998). Besides signaling for proteasomal degradation, polyubiquitination has been shown to activate phosphorylation (Pickart, 2001b). The ubiquitin chain can be formed by linkage through different lysine residues of ubiquitin. Usually Lys48-linked polyubiquitin chains target proteins for proteasomal degradation, while Lys63-linked ubiquitins regulate endocytosis and lysosomal sorting (Weissman, 2001).

The formation of the polyubiquitin chain to the substrate proteins requires the sequential action of three different enzymes (Figure 3). In the first step of this process, ubiquitin is activated in an ATP-dependent manner by the E1 enzyme. A single ubiquitin activating enzyme E1 is present in most organisms, including yeast and humans, and as ubiquitin is essential for life so is the E1 enzyme (McGrath et al., 1991). The activated ubiquitin is then transferred from E1 to the next enzyme in the cascade, the E2 conjugating enzyme (Hochstrasser, 1996).

Ubiquitin E3 ligase is the component of the ubiquitin conjugation pathway most directly involved with substrate specificity. However, it is also the least well-understood factor of the ubiquitin system. An E3 ligase is defined as an enzyme that binds, directly or indirectly, specific protein substrates and promotes the transfer of

ubiquitin from the E2 conjugating enzyme to the substrate protein or another ubiquitin on a polyubiquitin chain. Several different E3s, each recognizing a unique set of substrates, work in conjunction with a single or a few E2s (Hershko and Ciechanover, 1998). The E3s can be classified into two distinct families according to the catalytic domain they utilize, namely the *hect* domain E3s and the RING-finger family of E3s (Huibregtse et al., 1995; Deshaies, 1999). In both families, the E3 ligase binds both the substrate and the E2, and positions them so that the activated ubiquitin can be transferred from E2 to a lysine residue of the substrate protein (Hershko et al., 1983). Recently, additional types of E3s, such as U-box ubiquitin ligases, have been identified (Hatakeyama and Nakayama, 2003) and a subfamily of E3 ligases have been shown to also ubiquitinate non-lysine residues (Cadwell and Coscoy, 2005).

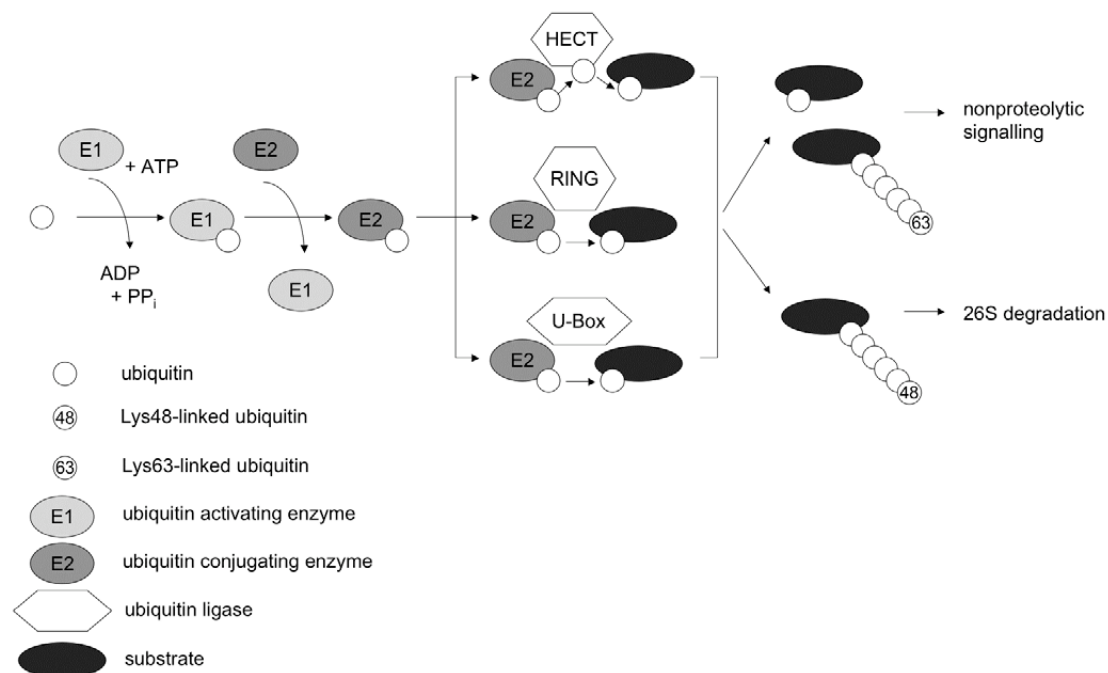


Figure 3. An overview of the ubiquitin conjugation pathway.

A ubiquitin-activating enzyme (E1) first activates ubiquitin in an ATP-dependent reaction. The activated ubiquitin then binds to ubiquitin conjugating enzyme (E2), which acts in conjunction with a ubiquitin E3-ligase. The E3-ligase binds to the protein substrates and promotes the transfer of ubiquitin from the E2 to the substrate creating a polyubiquitin chain and targeting the substrate protein for degradation or regulating lysosomal sorting or endocytosis. Adapted from Gao and Karin 2005.

The organization of the ubiquitin conjugation cascade is hierarchic. Whereas in most eukaryotes there is a single E1 enzyme, there are at least 13 E2 enzymes in yeast, and it's estimated that mammals might have as many as 60 different E2s (Hochstrasser,

1996; Gao and Karin, 2005). A vast number of E3s are present in all organisms, only a fraction of which have been identified thus far (Pickart, 2001a). As the E3s play a critical role in selecting the substrates, regulation of either the catalytic activity or the substrate interaction properties of the E3s allows the best degree of selectivity as well as the regulation of effectiveness of the ubiquitin cascade. Phosphorylation of either the substrate or the E3 have been shown to regulate ubiquitination (Gao and Karin, 2005).

Ubiquitinated proteins are in a dynamic state. They are either subject to further rounds of ubiquitination, ubiquitin removal by deubiquitinating enzymes or degradation by the 26S proteasome (Hochstrasser, 1996; Wilkinson, 2000). The 26S proteasome is a multisubunit complex that breaks down proteins into peptides and recycles ubiquitin. It belongs to the family of self-compartmentalized proteases, with regulatory units containing several ATP-dependent chaperones, and a cylindrical core proteinase (20S proteasome) capped by the regulatory units at both ends. The ATPases presumably help unfold the substrates and move them through the narrow pore leading to the proteolytic active sites (Baumeister et al., 1998). The substrates for proteasomal degradation are recognized through polyubiquitin chains (Pickart, 2001b).

3.2 TRIM proteins in disease

The TRIM family proteins are involved in a multitude of cellular processes, including apoptosis, cell cycle regulation, tumorigenesis and viral response. Consequentially, TRIM proteins are associated with several different pathological conditions varying from rare mendelian disorders to cancer and viral infections (Meroni and Diez-Roux, 2005).

Mendelian disorders resulting from mutations in TRIM proteins include, for example, familial mediterranean fever (FMF; OMIM# 249100), which is due to mutations in TRIM20, also known as Pyrin. FMF is an inflammatory disorder characterized by recurrent attacks of fever and polyserositis (Consortium, 1997a; Consortium, 1997b). Pyrin is an atypical TRIM protein as it lacks the RING domain. However, the organization of other motifs is typical for a TRIM domain and thus it is considered as a member of the TRIM protein family (Reymond et al., 2001). The function of this protein is as yet unknown. On the other hand, TRIM18, also known as

Midin, underlies X-linked Opitz syndrome (OS; OMIM# 300000), a complex congenital disease affecting mainly ventral midline development (Quaderi et al., 1997; De Falco et al., 2003). The *MID1* gene encodes a RING finger E3 ligase that ubiquitinates protein phosphatase 2A (PP2A), and the pathological phenotype is due to the diminished proteolysis of PP2A (Trockenbacher et al., 2001).

Changes in the expression of TRIM proteins are seen in different pathogenic conditions. For example, TRIM32, another TRIM protein with ubiquitin E3 ligase activity, is associated with skin carcinogenesis and is highly expressed in skin derived tumors (Horn et al., 2004). Further, some TRIM family members have been shown to be involved in innate cellular responses to viral infection. For example, TRIM5 has a splice form, TRIM5 α , which was shown to restrict HIV-1 infection in Old World Monkey cells, suggesting that TRIM5 may recognize and ubiquitinate the HIV capsid and target it for degradation (Stremlau et al., 2004).

3.3 The TRIM37 protein

The *TRIM37* gene underlying mulibrey nanism encodes a member of the TRIM protein family. In TRIM37, the N-terminal TRIM domain is followed by an internal TRAF (tumor necrosis factor (TNF) receptor associated factor) domain, and the C-terminal part of the protein has no known functional domains (Figure 4) (Avela et al., 2000). However, two aspartate-glutamate-serine (DES)-rich sequences are located in the C-terminal part of TRIM37, but apart from a weak homology to several transcriptional regulators, these have no known functions (Avela et al., 2000). Classical TRAF family proteins are involved in TNF receptor signaling. They serve as adaptor proteins for a wide variety of cell surface receptors, possibly by linking the cytosolic domain of the receptor to downstream protein kinases or ubiquitin ligases, and regulate apoptosis and cell stress responses (Arch et al., 1998; Deng et al., 2000).

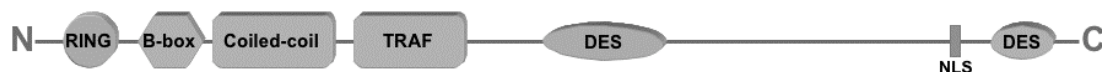


Figure 4. A schematic presentation of TRIM37.

The functional domains and the DES-rich regions are shown. Two adjacent nuclear localization signals are present at the position marked with NLS.

Unlike classical TRAF family proteins, in TRIM37 the TRAF domain is located in the central region of the protein rather than the C-terminal end. TRIM37 has been shown to be capable of binding to the classical TRAF family proteins and itself via its TRAF domain (Zapata et al., 2001).

3.3.1 TRIM37 localizes to peroxisomes

Two adjacent nuclear localization signals are present in the TRIM37 sequence, predicting nuclear localization for the protein (Figure 4). In immunofluorescence studies, however, both endogenous and ectopically expressed TRIM37 localize, at least partially, to peroxisomes classifying mulibrey nanism as a peroxisomal disorder (Kallijärvi et al., 2002). No known peroxisomal targeting signals are present in TRIM37, so the localization may be dependent on interaction partners. The coiled-coil region of the TRIM domain has been shown to be critical and sufficient to localize TRIM proteins to their correct subcellular compartments (Reymond et al., 2001). This is most likely also true for TRIM37, as mutant TRIM37 resulting from the Finnish major mutation that disrupts the coiled-coil region loses the peroxisomal localization, while the mutant protein resulting from the Finnish minor mutation with an intact TRIM domain targets correctly to peroxisomes (Kallijärvi et al., 2002). Interestingly, the TRIM proteins have been shown to interact with themselves and the coiled-coil region is necessary and sufficient for this interaction (Reymond et al., 2001). Since coiled-coil regions also mediate oligomerization of proteins in general by selective heterodimerization (Lupas, 1996), it may well be responsible for the peroxisomal localization of TRIM37 by interacting with an as yet unidentified protein that targets to peroxisomes.

Peroxisomes are single membrane-bound organelles that participate in a wide variety of metabolic processes in eukaryotic cells. An interesting property of peroxisomes is that their size, number, protein content and physiological function are adjusted according to the cell's metabolic and developmental state (Johnson and Olsen, 2001). Apart from their role in lipid metabolism and hydrogen peroxide detoxification, peroxisomes have also been shown to function in intracellular signaling and in regulating developmental processes (Titorenko and Rachubinski, 2004).

Improper assembly of peroxisomes results in metabolic or developmental defects and lethality or severe disease (Wanders, 2004). In general, peroxisomal disorders are divided into two subgroups including disorders of peroxisome biogenesis (PBD), in which the peroxisome is not formed properly, and single peroxisomal enzyme deficiencies (Wanders, 2004). Some examples of peroxisomal disorders are listed in table 3.

Mulibrey nanism shares several symptoms with other peroxisomal disorders, in particular with the PBDs. These include short stature, facial dysmorphism, retinal pigmentary changes, muscular hypotonicity and hepatomegaly (Karlberg et al., 2004a). However, the neurological phenotype is remarkably milder in mulibrey nanism than it is in most peroxisomal disorders with no notable changes in intelligence and only mild structural defects present in the central nervous system (CNS) (Karlberg et al., 2004a).

Table 3. Examples of peroxisomal disorders.

The Zellweger syndrome and rhizomelic chondrodysplasia punctata are PBDs while the X-linked adrenoleukodystrophy and rhizomelic chondrodysplasia punctata type 2 are single peroxisomal enzyme deficiencies.

Disorder (OMIM #)	Underlying gene (s)	Major clinical features	References
Zellweger syndrome (214100)	<i>PEX1, PEX2, PEX3, PEX5, PEX6, PEX12, PEX14, PEX26</i>	multiple congenital anomalies, patients rarely survive the first year of life	Brosius and Gartner, 2002
Rhizomelic Chondrodysplasia Punctata (215100)	<i>PEX7</i>	short stature, typical facial features, severe mental retardation	Agamanolis and Novak, 1995 Braverman et al., 1997 Motley et al., 1997 Purdue et al., 1997
X-linked adrenoleukodystrophy (300100)	<i>ABCD1</i>	neurodegenerative disorder, accumulation of very-long-chain fatty acids in plasma and tissues	Mosser et al., 1993 Gartner et al., 1998 Moser et al., 2000
Rhizomelic Chondrodysplasia Punctata type 2 (222765)	<i>DHAPAT</i>	short stature, developmental delay	Wanders et al., 1992 Ofman et al., 1998

3.3.2 TRIM37 has ubiquitin E3 ligase activity

The physiological function of TRIM37 is unknown. However, as for some other members of the TRIM protein family, autoubiquitination and formation of poly-ubiquitin chains has also been reported for TRIM37, suggesting that it acts as a

ubiquitin E3 ligase. Thus, it may be involved in proteasomal protein degradation or signaling (Kallijärvi et al., 2005). No substrate proteins for TRIM37 are known, and the identification of these would be important as the accumulation of these substrate proteins may underlie the pathogenetic mechanisms behind mulibrey nanism. TRIM37 is highly insoluble, and when overexpressed it forms aggregates that have been characterized as aggresomes (Kallijärvi et al., 2005). Aggresomes are aggregates of nondegraded proteins that form in the pericentriolar region when the production of misfolded proteins exceeds the capacity of the proteasomal degradation machinery (Johnston et al., 1998). Thus, the accumulation of overexpressed TRIM37 to aggresomes suggests that TRIM37 may itself be degraded through the proteasome pathway.

TRIM37 is the first peroxisomal ubiquitin E3 ligase identified in mammals, and whether ubiquitin exists inside peroxisomes is not known. However, in yeast a peroxisomal membrane protein, Pex4p, acts as a ubiquitin conjugating enzyme (Crane et al., 1994) and recently ubiquitin dependent localization and recycling between peroxisomes and the cytosol was reported for a yeast Pex20p protein (Leon et al., 2006). Whether the ubiquitin E3 ligase activity of TRIM37 is a peroxisomal function is not known.

Insulin signaling is a transitory effect that needs rigorous control in order for the cells to maintain their homeostasis (Rome et al., 2004). Correct levels of different signaling molecules are important for proper insulin action, and altered levels of key molecules have been associated with type 2 diabetes and other insulin related pathologies. The levels of these signaling molecules are controlled by their synthesis and degradation rates. The ubiquitin-proteasome pathway is one of the major proteasomal degradation pathways, and has been suggested to affect the regulation of the insulin signaling cascades and insulin action at several levels (Rome et al., 2004). Thus, as TRIM37 has ubiquitin E3 ligase activity, and loss-of-function mutations result in IRS, it is tempting to speculate that TRIM37 might have a role in regulating insulin signaling.

4. Regulation of gene expression

Expression of mammalian genes can be regulated on spatial and temporal levels. Some genes are expressed essentially in all cell types and at all times. These genes are called housekeeping genes and often encode proteins required for essential and general functions of a cell. However, some genes show very restricted expression patterns and are expressed only in a specific tissue, in some individual cells, at a specific developmental stage or at specific times of the cell cycle (Strachan and Read, 2003). For example, during development and embryogenesis coordination and tight regulation of gene expression is essential for proper division and differentiation of individual cells. Expression has to be up or down regulated during certain critical periods or above or below certain critical thresholds for the proper developmental processes to occur and give rise to the specialized tissues and organs of an organism (Gilbert, 2000). Further, for some genes the default stage is inactive and an inducer is needed for their expression. Ectopic expression, i.e. expression in an improper place or at improper time, can have devastating effects, thus tight regulation of timing, location and level of expression are essential for several genes (Strachan and Read, 2003).

Control mechanisms regulating gene expression are highly conserved among different species. In prokaryotes, two major levels of gene regulation have been described, one at the chromatin level and the other involving RNA polymerase. First of all, for a gene to be expressed the DNA has to be accessible. In its normal state, DNA is tightly packed around a set of histones, resulting in a condensed inaccessible state. Before a gene can be transcribed this condensed form has to be unraveled. Histone modifications, methylation, acetylation, phosphorylation and ubiquitination play a crucial role in controlling chromatin condensation (Berger, 2002). When the DNA is accessible, RNA polymerase can then catalyze the DNA-dependent synthesis of mRNA, and the specificity and effectiveness of the polymerase regulate the rate of mRNA synthesis. Analogous but more complex mechanisms work in eukaryotes, and some additional control mechanisms have been observed in mammals (Kornberg, 1999).

4.1 Transcriptional control

Several different mechanisms regulate mammalian gene expression at the transcriptional level and the major control point of gene regulation occurs at the initiation of transcription (Young et al., 2002).

When the DNA is accessible, co-operation of several different factors are needed for transcription to start. The *cis*-acting promoter is one of the major elements. The core promoter region, located near the transcription initiation site, contains the basal promoter elements which bind proteins that belong to the general transcription machinery. Upstream from the transcription initiation site are the positive or negative proximal promoter elements, namely enhancers or silencers. Trans-acting sequence specific activator- and repressor- proteins bind to these specific sequences and either increase or decrease the basal expression level. Further away from the core promoter region are the distal enhancer elements that can be located thousands of base pairs away from the actual gene they are regulating. The general transcription machinery that contains RNA polymerase II and general transcription factors is sufficient to promote basal gene transcription (Goodrich et al., 1996), and recruitment and binding of these elements to the promoter region as well as the effectiveness of the polymerase are major players in initiating transcription (Young et al., 2002). However, several sequence-specific transcription factors and different co-regulatory proteins are required for efficient transcription and for specific regulation of expression (Orphanides and Reinberg, 2002; Woychik and Hampsey, 2002).

4.2 Translational control

Translation of mRNA into a protein is the final step in the gene expression pathway, and regulation of translation is one mechanism to control gene expression (Gebauer and Hentze, 2004). Translational control can have much more rapid effects on the protein level than does transcriptional control, and can thus be used to fine-tune protein levels. Although most of the regulation of gene expression takes place at the transcriptional level, translational control is crucial especially in early embryogenesis and also at later developmental stages including, for example, sex determination (Kuersten and Goodwin, 2003). Global control of translation that affects all translation occurring at a specific time in a specific cell is mainly achieved through modification of translation initiation factors. Gene specific regulation, on the other

hand, is driven by regulatory protein complexes or mRNA molecules that bind to specific sequences, usually present at the 5' or 3' untranslated regions (UTR) of these genes (Gebauer and Hentze, 2004). Thus, regulatory sequences and structural features of the mRNA are responsible for its translational fate. The cap structure and the polyA tail are strong promoters of translation initiation, upstream open reading frames (uORFs) often reduce translation of the main ORF, and several secondary or tertiary structures, for example hairpins, normally block translation initiation. Most of the regulatory elements discovered so far are inhibitory, implying that mRNAs are translationally active by default (Gebauer and Hentze, 2004).

The 3' UTR is a major functional part of the mRNA responsible for translational regulation (Kuersten and Goodwin, 2003; de Moor et al., 2005). The length of the polyA tail has an effect on translation as long polyA tails correlate with translation, while short polyA tails correlate with repression of translation (de Moor et al., 2005). Different control elements in the 3' UTRs also regulate turnover rates and localization of mRNAs in cells (Kuersten and Goodwin, 2003; Parker and Song, 2004).

Translational regulation can also occur through small microRNAs (miRNAs), which bind specific sequences at the 3' UTRs and repress translation (Filipowicz et al., 2005). Recently, the use of small interfering RNAs (siRNAs) has proven to be an effective method of repressing translation experimentally. However, there is a functional difference between endogenous miRNAs and synthetic siRNAs, based on the degree of complementarity. While miRNAs bind by incomplete base-pairing, siRNAs have a perfect match to the target mRNA and repress translation by degrading mRNA (Hutvagner and Zamore, 2002; Filipowicz et al., 2005).

4.2.1 Translational control in germline

Formation of the germ cell lineage is critical for survival of a species. Germ cells are the only cells that contribute genetically to the next generation and they are the only cells undergoing meiosis and recombination. Thus, integrity of the germ cell genome is extremely important. Precise timing and regulation of expression of a wide variety of genes is essential for mammalian meiosis and germ cell development, and this is mainly achieved through translational control (Eddy and O'Brien, 1998). The regulation can be positive, allowing translation of proteins necessary for germ cell

specification, or negative, repressing translation of proteins promoting somatic fate (Leatherman and Jongens, 2003).

Many genes have atypical patterns of expression during meiosis. Some of the developmentally regulated genes are transcribed exclusively in germ cells and some genes that are also expressed in somatic cells produce germ cell specific transcripts (Eddy and O'Brien, 1998). For example, a RING finger protein, RNF4, has a testis-specific transcript present specifically in spermatids. The transcript encodes a protein product identical to that present in other tissues but differs from the major transcript only in the 3' untranslated region. This testis specific RNF4 transcript is translationally repressed and only a small fraction of it is translated (Pero et al., 2003).

4.3 Naturally occurring antisense RNAs

Natural antisense RNAs are endogenous transcripts with complementary sequences to other transcripts named sense transcripts. Most are *cis*-encoded, that is, encoded from the same locus and transcribed from opposite strands. In contrast to *cis*-encoded transcripts that display perfect complementarity to sense transcripts, *trans*-encoded antisense transcripts may show only partial complementarity, since they are transcribed from a different locus (Vanhee-Brossollet and Vaquero, 1998).

Antisense transcripts were first identified in prokaryotes, where they serve as a mechanism to regulate gene expression (Wagner and Simons, 1994). Recent studies have shown that the presence of naturally occurring antisense RNAs is a common feature also in the human genome (Lehner et al., 2002; Yelin et al., 2003), and growing evidence for coupled, balanced or unbalanced, expression of counterpart genes indicates a role for antisense transcripts in regulating expression of their sense counterpart genes in humans (Vanhee-Brossollet and Vaquero, 1998; Luther, 2005). This regulation can take place either at the transcriptional or translational level and, in addition, the maturation, transport or stability of the mRNA can be affected (Vanhee-Brossollet and Vaquero, 1998). Negative down-regulation has generally been suggested, but positive up-regulation by stabilization of the sense transcript is also conceivable.

Two possible mechanisms for antisense mediated regulation have been suggested. Due to the similar structure, antisense mRNA may bind to proteins interacting with sense mRNA, and thus deprive the sense transcripts from proteins

necessary for their expression (Kim et al., 1996; Lehner et al., 2002). Another mechanism is analogous to the effects of micro- or short interfering RNAs, where an antisense RNA binds to the sense counterpart and forms a duplex. Two plausible consequences for this duplex formation have been suggested. The double stranded RNAs can either be degraded by double-stranded RNA (dsRNA) specific enzymes (Hammond, 2005), or steric hindrance can prevent RNA interacting with molecules that are required for expression (Vanhee-Brossollet and Vaquero, 1998).

4.4 Alternative splicing

As the human genome project produced more sequence and the number of identified genes grew, the estimated total number of genes present in the human genome declined remarkably. The current estimate of the total number of genes in the human genome is only 20 000 to 25 000 (Human Genome Project; <http://www.ornl.gov/>), not significantly more than the estimated 20 000 genes in the worm genome (Hodgkin, 2001). However, while the estimated total number of genes has been reduced, the number of genes known to undergo alternative splicing has expanded. To date, it is estimated that 60% of all human genes and 74% of multi-exon genes are alternatively spliced (Modrek and Lee, 2002; Johnson et al., 2003). Thus, alternative splicing explains how the vast proteomic complexity is achieved with a limited number of genes.

Alternative splice variants can be produced through the use of alternative promoters, resulting in differently expressed variants of the same gene (Stamm et al., 2005). The majority, approximately 75%, of alternative splicing events, however, affect the protein coding regions of the genes (Zavolan et al., 2003). New parts can either be included or existing parts removed from the protein product. Changes in the protein primary structure can affect binding properties, stability or intracellular localization of the protein. Post-translational modifications that the protein is subject to can also vary. Further, both soluble and membrane bound forms of the same protein can be produced through alternative splicing (Stamm et al., 2005).

A conserved surveillance mechanism, named nonsense-mediated mRNA decay (NMD), monitors mRNAs for errors and degrades those containing premature termination codons (PTCs) (Hentze and Kulozik, 1999). Several different mechanisms by which the NMD machinery operates have been proposed and the

current general view involves intron positions as markers for recognition of PTCs and cytoplasmic translation as a signal to elicit NMD (Schell et al., 2002). Most pre-mRNAs undergo processing by capping, splicing and polyadenylation. When splicing machinery removes introns, some components of the machinery, namely the exon junction complex (EJC), remain at the splice junctions of the mature transcript (Le Hir et al., 2000). In one of the proposed mechanisms for NMD during the first round of translation ribosomes remove the EJCs, but as the ribosome halts at the translation termination codon EJCs downstream of the termination codon are not removed. The remaining EJCs recruit Upf proteins which in turn interact with the translation termination factors and trigger the NMD process (Thermann et al., 1998; Baker and Parker, 2004). Hundreds of human genetic disorders are influenced by this mechanism, since about 25% of all known human mutations produce PTCs and are predicted to trigger NMD (Culbertson, 1999).

Recently, alternative splicing has emerged as an important mechanism regulating not merely protein diversity, but also protein expression (Lareau et al., 2004; Stamm et al., 2005). Introduction of PTCs to mRNAs through alternative splicing has been shown to be a relatively common feature of the human transcriptome, with 25% to 35% of alternative exons introducing frameshifts or premature stop codons into the pre-mRNA (Stamm et al., 2005). Since PTCs expose mRNAs as targets for NMD, alternative splicing provides a mechanism to regulate protein expression through unproductive splicing and mRNA surveillance (Lewis et al., 2003; Hillman et al., 2004). Further, nonsense surveillance is not only limited to premature termination codons, but NMD degrades a variety of aberrant mRNAs by recognizing alterations in normal spatial relationships between the termination codon and other pre-mRNA features (Baker and Parker, 2004). Lately, a microarray-based approach was used to demonstrate that NMD regulates hundreds of physiologic human transcripts, and not just those containing nonsense mutations, supporting the idea that the predominant role for NMD is to regulate expression levels of a wide variety of physiologic mRNAs rather than just modify disease caused by nonsense mutations (Mendell et al., 2004; Alonso, 2005). It has been estimated that at least one-third of all alternatively spliced mRNAs are downregulated by NMD (Maquat, 2005).

AIMS OF THE STUDY

1. To characterize the *TRIM37* gene in detail by characterizing the genomic structure of the gene and identifying alternative splice variants and the regulatory promoter region
2. To identify and characterize mutations in *TRIM37* underlying mulibrey nanism
3. To characterize the mouse *Trim37* gene
4. To study the tissue expression of TRIM37 in humans and mice

MATERIALS AND METHODS

Thirtyone recently diagnosed mulibrey nanism patients of different ethnic backgrounds were included in the mutational analysis of this study. The patients were diagnosed by local clinicians, and some of them fulfill the diagnostic criteria of mulibrey nanism, while some showed just short stature and some mulibrey-like features. Additionally, thirtyfive Finnish patients, with short stature and some mulibrey-like features were analysed. All samples were obtained after informed consent was received from the patients or their guardians. The control samples were from the Centre d'Etude du Polymorphisme Humain (CEPH). Studies including patients have been approved by the Ethics Committee for the Department of Medical Genetics, University of Helsinki.

The methods used in the original articles included in this thesis are summarized in Table 4.

Table 4. Methods used in this study

Method	Original publication
Mutation analysis	I, II
Site-directed mutagenesis	I, II
DNA and RNA extraction	I, II
DNA sequencing	I, II, III, IV
Recombinant DNA techniques (cloning)	I, II, III
Gel electrophoresis (PAGE, SSCP)	I, III
Primer extension	III
Quantitative real-time PCR	III
Cell culture	I, II, III
Transient transfections	I, II, III
Immunofluorescence staining	I, II
Immunofluorescence microscopy	I, II
Northern blot analysis	III, IV
Southern blot analysis	I
Western blot analysis	I, II
Luciferase-reporter assays	III
Immunohistochemistry	IV
Production of GST-fusion proteins	II
Immunoprecipitation	II
<i>In vitro</i> ubiquitination assays	II
Database and computer analysis	I, II, III, IV

RESULTS AND DISCUSSION

1. Characterization of the *TRIM37* gene (I, III, unpublished)

1.1 Genomic structure (I, III, unpublished)

Before this study the *TRIM37* gene had been identified as the causative gene underlying mulibrey nanism. However, at that time only a 4111-bp cDNA sequence (KIAA00898) was available, and this study begun by determination of the complete genomic structure of *TRIM37*. At that time the human genome project was ongoing and a vast amount of genomic sequence had already been produced and was readily available in public databases. However, the sequence was not complete and many gaps existed. A search through the public databases revealed two genomic sequences covering the 5' (nts -273-684; exons 1-8) and 3' (nts 810-4111; exons 10-24) ends of the *TRIM37* gene. No genomic sequence was available for a 125-bp part of the cDNA sequence (nts 685-809; exon 9) at that time. The database genomic sequences were organized according to the cDNA sequence. To fill the remaining gap, PCR-amplified products between the flanking genomic sequences and the cDNA sequence between them were sequenced. This enabled us to assemble a single genomic sequence covering the whole *TRIM37* genomic region, including nts 685-809, with only minor intronic gaps. Later, when the human genome project produced more sequence, the genomic sequence we assembled was confirmed and completed with a novel chromosome 17 genomic sequence contig (NT_010783).

By aligning the cDNA sequence to the assembled genomic sequence, we determined the genomic size of *TRIM37* to be approximately 109 kb. The *TRIM37* gene was initially found to have 24 exons. Later an additional 25th exon was found and the full genomic size was extended to approximately 125 kb. The sizes of the exons range from 41 to 309 bp, and the introns from 1.2 to 16.5 kb. All the splice junctions follow the conserved GT-AG rule. The sequences of the exon-intron boundaries and the sizes of all the exons and introns are shown in Table 5. A schematic presentation of the structure of *TRIM37* is shown in section 2 in Figure 7.

Table 5. *TRIM37* exon-intron boundaries.

The coding exonic sequences are shown in capital letters while the non-coding intronic sequences are in small letters.

Exon nr	Exon size (bp)*	3'Splice Acceptor Site	5'Splice Donor Site	Intron nr	Intron size (kb)
1	294 (21)		GAGCGTGGAGgtgagggggt	1	2.0
2	102	ctgttttcagAGCATTGCTG	CTGTATTAGGgtaagttgta	2	13.0
3	41	gcccccttagCGCTGGCTGA	CTCATTGCCGgtaagtgttt	3	2.9
4	117	tttccccagTGCTCCACTC	AAAAGGACAAgtatgtcctc	4	4.2
5	88	taatgtatagATGTGAAAAT	GGGAGGAATGgtgagcagaa	5	2.8
6	123	atttaaataagCATGGCGGAC	TCAAGAAGTGgtaagactac	6	1.2
7	124	tatcttctagGAAAGGAATG	ACACTGATGGgtaagtgttt	7	4.0
8	68	ttttcatcagGTCAGAAAGAC	GGAGCACCAGgtgatgaaat	8	5.1
9	125	cctcacacagTTGCGGTCTT	ACTTTACCAGgtatgacaca	9	6.6
10	51	tttttttagTGAATTAGTG	AGAATTTTCAGgtaagagttt	10	1.7
11	82	ttttcgtagCACTTTGCGT	AGTTTACCCAgtaagttttt	11	1.5
12	77	cttcttttagGATGGAAATG	AAACTTCTAAgtaagaagtg	12	4.0
13	180	tcaattttagATATGAATAT	TGATTTTAAGgtaacaggaa	13	5.5
14	115	ttctttaagGTTTCAGGTA	CCTTAAAGAGgtaagaaaat	14	1.8
15	216	gttttaaagAGACTTACTA	AGATTATCATgtaataatga	15	1.4
16	137	attgatctagCACGAGCTTT	AAGAAACTATgtgagtttcc	16	5.8
17	86	atattcttagGTCTGGAGAA	GGACCCGCAGgtaatgaggt	17	9.7
18	195	tcttttcagGTAGTAGCCA	CAGCCCACAGgtaaaataat	18	3.2
19	309	ttctctgaagCATCATATTC	ATACGAAACTgtaagtaata	19	11.0
20	129	gtctttgcagCCACAAATAA	CTGTCTGAAGgtaaattga	20	1.5
21	190	tttttgagGCTCCCCAGG	TCACCTTGGGgtaaattaag	21	3.2
22	119	cttctttagGGCTAATGCT	CCTGAAGAAGgtaaggaaatg	22	10.6
23	117	ttcctgccagGAATGAGTAG	CCGGATGAAGgtgcagcaca	23	2.1
24	79/>1254	ttaatcatagATACACATTC	ATAGTGAAGgtaattgcca	24	16.5
25	>284	gttttcttagATAATTTGAT			

*The coding region of exon 1 is shown in parentheses and two different forms of exon 24 are presented representing the alternative splice forms of this exon. Exon 25 is present only in the splice form with the shorter exon 24 (see section 1.2.1).

1.2 Alternative splicing (III)

Northern analysis of the *TRIM37* gene shows a 4.5-kb transcript present in all tissues studied, and an additional 3.6-kb transcript in testis (Avela et al., 2000). Further, amplification of the *TRIM37* coding region by RT-PCR resulted in several alternative products of different lengths. To identify alternative splice variants of *TRIM37*, with special emphasis on the shorter transcript seen in testis, a BLAST search was run of

the human EST database. The search returned 211 sequences derived from several different tissues. Among these EST sequences, 12 alternatively spliced forms of *TRIM37* were found (Table 6). RT-PCR experiments confirmed these alternative sequences and revealed 18 additional variants (Table 7). Most of these 30 alternative variants predict non-functional protein products, 13 by deleting functional domains and 11 by producing premature termination codons (PTCs), while two predict in-frame insertion of 9 or 16 aas into the polypeptide.

Table 6. Alternative splice variants of *TRIM37* identified from the EST sequences and confirmed with RT-PCR.

EST clone(s), tissue	Difference to NM_015294	Predicted protein product
BC036012, AW589999, testis	<i>Trim37b</i> #	964 aa identical to TRIM37
AK025648, hepatoma	<i>Trim37a</i> , del exon 23	925 aa TRIM and TRAF intact
BM979745, lung	<i>Trim37b</i> , del exon 23	925 aa TRIM and TRAF intact
AI307801, kidney	<i>Trim37b</i> , exon 23 ins27	973 aa TRIM and TRAF intact
BE703384, brain	additional exon 8B (48 nt)	980 aa TRIM and TRAF intact
AA460052, AA460884, total fetal	exons 1-10 + intron 10	287 aa TRAF domain missing
BI913823, brain	exons 1-4 + intron 4	121 aa only RING domain left
BG721833, testis	del exon 2	930 aa RING domain missing
BG997038, head/neck	del exon 5	113 aa PTC*
BG220351, fibrosarcoma	del exon 8	209 aa PTC
BX537955, testis	additional exon 3B (76 nt)	67 aa PTC
BI831450, brain	additional exon 3B + intron 4	67 aa PTC

See section 1.2.1 for details of *TRIM37b*

* PTC = premature termination codon

Table 7. Alternative splice variants of *TRIM37* identified by RT-PCR.

mRNA template tissue	Difference to NM_015294	Predicted protein product
testis	del exons 5-12	718 aa only RING domain left
testis	del exons 5-13	658 aa only RING domain left
lymphoblast	del exons 5-15 and part of exons 4 and 16 (c.273_1649)	505 aa only RING domain left
heart	del exons 5-17 and part of exon 4 (c.271_1758)	468 aa only RING domain left
testis	del exons 4-16	463 aa only RING domain left
testis	del exons 14-23 and part of exon 13 (c.1062_2810)	381 aa TRIM and TRAF intact
testis	del exons 3-17 and part of exons 2 and 18 (c.57_1892)	352 aa TRIM and TRAF missing
heart	del exons 10-22 and part of exons 9 and 23 (c.808_2718)	327 aa, TRAF domain missing
heart	del exons 12-21	317 aa PTC
placenta	del exons 11-23	293 aa TRAF domain missing
testis	del exons 10-23	276 aa TRAF domain missing
placenta	del exons 5, 8-13, 15-16, 18-19, 21, and 23	113 aa PTC
placenta	del exons 5-18 and part of exons 4 and 19 (c.262_2075)	111 aa PTC
testis	del exons 5-23	100 aa only RING domain left
heart, testis, placenta, lymphoblast	del exons 5-19	96 aa PTC
testis	del exons 9-23, additional exon 2B (188 nt c.123+102_123+289)	68 aa PTC
testis	del exons 3-19, ins part of intron 22 (c.2696-41_2691-1)	56 aa PTC
testis	del exons 4-22 and part of exons 3 and 23 (c.157_2714)	53 aa PTC

Both *in silico* analyses and RT-PCR experiments showed that alternative splicing of *TRIM37* is relatively frequent. However, most of the alternative *TRIM37* transcripts seem to be expressed transiently and at fairly low levels. They are not seen in other species and RT-PCR favors the major transcripts over them. Further, many of them predict either non-functional or truncated protein products. The relatively frequent occurrence of seemingly non-functional splice variants suggests that alternative splicing has a role in regulating *TRIM37* expression post-transcriptionally through regulated unproductive splicing and translation and nonsense surveillance (Lewis et al., 2003). The *TRIM37* gene is fairly large (~125 kb), thus the transcription is time consuming and if rapid changes on the protein level are needed, translational regulation of expression has much more prompt effects than transcriptional regulation. A similar phenomenon is seen for example for *TRIM18* (also known as

MIDI) underlying OS, where alternative splicing is thought to regulate *MIDI* function translationally by producing variants that predict loss-of-function via introduction of PTCs or altered interaction properties of the splice isoforms. This is further thought to underlie the tissue specific function of *MIDI* and the fact that the clinical features of OS are confined to the developing ventral midline, while *MIDI* mRNA is ubiquitously expressed (Winter et al., 2004).

Only four of the alternative *TRIM37* transcripts seem to be expressed constantly (Figure 5). *TRIM37a*, the database transcript, *TRIM37b*, a testis specific variant encoding a protein product identical to *TRIM37a* (see next section 1.2.1 for details), and in addition both of these two major transcripts have variants that lack exon 23. The skipping of exon 23 predicts an in-frame deletion of 39 amino acids, including an evolutionarily conserved DES (aspartate-glutamate-serine)-rich motif near the C-terminus (Figure 6). Whether the DES-rich region has any functional relevance is not known, and thus the physiological function of this transcript or whether it has any functional role is unknown. However, the transcripts lacking exon 23 were readily amplified by RT-PCR, suggesting high-levels of constitutional expression.

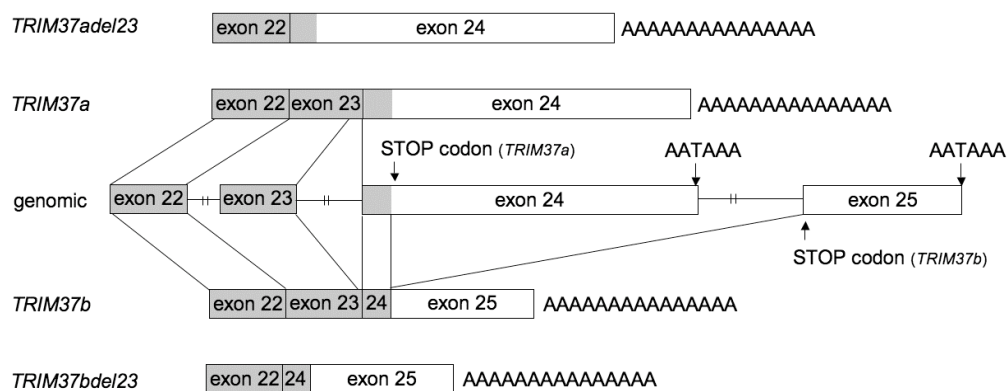


Figure 5. The four major alternative splice variants of *TRIM37*.

Structure of the 3' ends of the four constantly expressed *TRIM37* transcripts. The genomic structure of the *TRIM37* gene downstream of exon 22 is shown in the middle. The *TRIM37^a* and *TRIM37^b* transcripts encode identical protein products but use different termination codons and have different 3' untranslated sequences. In the *TRIM37^a* transcript the whole exon 24 sequence is included whereas in the *TRIM37^b* transcript, only the first 79 nucleotides of exon 24 are included followed by an additional exon (exon 25), predicting a shorter transcript (~3.6 kb). Both *a* and *b* forms have additional variants where exon 23 is deleted. Coding regions are shown in grey and untranslated regions in white.

human	SETGELQPYLPEGASAAPEEGHSSDSIDIECDTENEEQEEHTSVG-GFHDSEFMYMTQPPDEDTHSSFPDGEQIGPEDLSFNNTDENSGR
dog	SETGELQPYLPEGASAAPEEGHSSDSIDIECDTENEEQEEHTSMG-GFDDSFMA--QPPDEDTHSSFPDGEQIDPEDLSFNNTDGNSSGR
mouse	SEAGEYQPTLPEGASAAPEEGHSSDSIDIECDTENEEQEEHTSMG-AFNDPFLA--QPPDEDTHSSFPDGEQIDPENLHFNPDGEGGR
chicken	SETGELQGVLPPEGASAGPEEGHSSDSIDIECDTENEEQEDATSTSEGFNNAFVYQSSSEASERCSAFPGGDDVGTDDLSEVNGEDTTR
zebrafish	SSSHPDEGVLSQKPHTCVAQH-ENGHELDICDTENENEDEVVPLEAGDTSAF-----DITRLPCTGEQLIPDDLSTFAEESTER
Consensus	Se.ge.#gVLP#gasa.pe#gnssds#i#CDTENE####.ts...Gf...aF.....#t.s.fp.G#Q..p#DLSF...E.t.R

Figure 6. Alignment of the C-terminal parts of human, dog, mouse, chicken and zebrafish Trim37 proteins. The 39 amino acid region containing the DES-rich region deleted in the transcript that lacks exon 23 is boxed.

When transiently over-expressed, the TRIM37^{adel23} protein showed vesicular staining compatible with the peroxisomal localization of the full length protein, implicating that the 39 amino acid deletion does not result in altered subcellular localization. This is in line with previous studies showing that the coiled-coil region is responsible for subcellular localization in TRIM proteins (Reymond et al., 2001).

Expression levels of *TRIM37a* and *TRIM37b* were further studied with quantitative real-time PCR. As expected from the northern analysis, expression of *TRIM37* was seen in all tissues studied, with highest expression in testis, where both transcripts showed equal expression. High expression of *TRIM37a* was also seen in brain (both fetal and adult), whereas *TRIM37b* was detected mainly in testis. Brain and testis were the most abundant tissue sources also among the database *TRIM37* sequences, with 32 clones from brain and 19 clones from testis. In most of the tissues analysed, fetal tissues showed slightly higher expression levels than adult tissues.

1.2.1 *TRIM37b* (III)

Among the alternative splice variants predicting functional protein products was a testis-specific transcript, which we named *TRIM37b*. This transcript has an additional 25th exon, 16 kb downstream of exon 24 (Figure 5). In exon 24 a conserved splice donor site precedes the termination codon (TAA), with the intronic GT-sequence of the splice donor partly overlapping the termination codon (GTAA). However, in most cases this splice junction is not used. In testis, this donor site is activated, which in turn activates a novel splice acceptor site 16 kb downstream, and the product of this splicing event is *TRIM37b*. A conserved AG consensus sequence is present at the splice acceptor site in exon 25, where nucleotides two to four encode a termination codon substituting the exon 24 termination codon of *TRIM37a* at the identical position. Thus, these two transcripts predict identical protein products.

Among the identified *TRIM37* variants, *TRIM37a* and *TRIM37b* were the only transcripts with homologous variants seen also in other species (mouse and rat), implicating functional relevance for these isoforms.

The *TRIM37b* transcript was further shown to correspond to the 3.6-kb testis-specific fragment seen in northern analysis using 3' sequence specific probes. Hybridization of northern blots with several different probes from the coding region detected both the 4.5- and the 3.6-kb transcripts (publication III; Figure 4). On the contrary, hybridization with a *TRIM37a* 3' UTR probe (c.2920-3499) detected only the 4.5-kb transcript. Hybridization with a *TRIM37b* exon 25 probe resulted in several differently sized transcripts including the 3.6-kb *TRIM37* transcript. The other transcripts seen with this probe were identified as representing *PPM1E* (NM_014906). The *PPM1E* gene resides on the complementary strand and has 3' UTR sequence that overlaps with exon 25 of *TRIM37*. It encodes a protein phosphatase 1E, a protein belonging to the PP2C family of Ser/Thr protein phosphatases that act as negative regulators of cell stress response pathways. PPM1E inhibits actin stress fiber breakdown and morphological changes driven by cell division cycle 42 (CDC42) (Koh et al., 2002).

The 3' UTR sequence has several functions in regulating gene expression. It can affect mRNA stability, localization of the mRNA molecules in a cell, and provides the basis for translational control by harboring specific regulatory sequences that bind regulatory proteins (Wickens et al., 1997). Thus, the function of transcripts with alternative 3' UTRs coding for identical protein products is most likely regulatory. The presence of *TRIM37* transcripts with alternative 3' sequences specifically in testis implies that *TRIM37* expression is strictly regulated there. A similar phenomenon is seen for *RNF4*, another RING finger protein that has a translationally repressed testis specific transcript (Pero et al., 2003). The *PPM1E* gene on the complementary strand may also provide a mechanism through which *TRIM37* expression is regulated in testis. Transcribed from the opposite strands, *TRIM37b* and *PPM1E* could act as *cis*-encoded antisense transcripts and downregulate each other's expression.

Immunohistochemical studies on mouse tissues showed that Trim37 expression in testis is limited to developing spermatids (see section 4, Figure 11) and strict regulation of gene expression is known to be extremely important in germ cells

(Braun, 1998; Eddy and O'Brien, 1998). Thus, as mulibrey nanism patients are subfertile (Lipsanen-Nyman, 1986; Karlberg et al., 2004b), this may well be specifically due to TRIM37 dysfunction in developing germ cells.

1.3 Transcription initiation and promoter region (III)

To identify the transcription initiation site for *TRIM37*, a primer extension assay was performed. Several products corresponding to transcription start sites within a window of approximately 130 nts between nts -373 to -246 upstream of the ATG codon of the translation initiation site were produced.

To identify the promoter region of *TRIM37*, 5' flanking sequences of human, mouse and rat genes were aligned and run through different promoter prediction programs. The *in silico* analysis predicted an active promoter region starting from the translation initiation site and continuing 600 nucleotides upstream. This putative promoter sequence showed high homology to mouse (71%) and rat (72%) 5' flanking sequences. Several transcription factor-binding sites in this region were predicted to be present in all three species.

To assess whether the putative promoter region has promoter activity, different segments of the region were cloned in front of a promoterless luciferase reporter gene. All reporter constructs showed approximately 400 times the activity of the empty pGL3-basic vector, indicating that the elements essential for promoter activity are present in the shortest segment (nt -591 to -246). As the construct with the longest 5' sequence in inverse orientation showed only one fourth of the activity of the construct with the same sequence in forward orientation, the promoter activity seems to be orientation dependent.

Thus, the functional analyses confirmed the *in silico* prediction of a 600-bp promoter and showed that nucleotides -591 to -246 relative to the translation initiation site harbor the elements sufficient for strong basal activity. This promoter region is G+C rich (70%) and TATA-less, showing features typical of a house-keeping gene. However, as the protein expression shows a strictly regulated cell-specific pattern, this further suggests a role for translational regulation in controlling TRIM37 expression. The TATA-box plays a critical role in determining the site for transcription initiation and several examples exist of TATA-less promoters that lack a unique transcription start site. Instead, these TATA-less promoters have multiple,

sometimes widely dispersed sites of transcription initiation (Reynolds et al., 1985; Geng and Johnson, 1993). This seems to be true also for *TRIM37*, where several transcription initiation sites are dispersed in a window of 130 nts.

2. Identification of mulibrey nanism associated mutations (I, II, unpublished)

Characterization of the genomic structure of *TRIM37* allowed mutation screening for patients for whom no RNA was available. We amplified and sequenced all exons and the putative 600 bp promoter region with flanking intronic primers and consequently detected fourteen novel changes in eleven patients, two of these present in the same disease chromosome (Table 8).

Table 8. Mutations of the eleven mulibrey nanism patients included in this study

Origin of patient	Mutations	Reference
Canadian	p.[Gln249X]+[Met347fs]	I
Canadian	p.[Gly322Val]+[Arg471X]	I
Tunisian	p.[Arg471X]+[Arg471X]	I
Sicilian	p.[Arg439fs]+[Arg439fs]	I
Saudi-Arabian	p.[Arg686X]+[Arg686X]	I
Australian	p.[Cys109Ser]+[Glu271_Ser287del]	II
French	p.[Cys28fs]+[Cys28fs]	unpublished
German	p.[Lys411fs]+[Lys411fs]	unpublished
Swiss-Finnish*	p.[Arg166fs]+[Gln638fs]	unpublished
Turkish	p.[Glu632fs;Ala133Val]+[Glu632fs;Ala133Val]	unpublished
Finnish*	p.[Arg166fs]+[Tyr389X]	unpublished

*These two patients were compound heterozygotes for a novel mutation and the Finnish major mutation (p.Arg166fs) identified by Avela et al. 2000.

Like the previously identified mutations in *TRIM37*, most of the new mutations predict premature termination codons. Four of the new mutations were transitions, c.745C>T, c.1166A>G, c.1411C>T and c.2056C>T, leading to nonsense mutations and premature stop codons p.Gln249X, p.Tyr389X, p.Arg471X and p.Arg686X, respectively, and thus predicting either truncation of the protein products or mRNA degradation through NMD.

Five novel changes were small insertions or deletions. Two were one nucleotide deletions (c.81delG and c.1233delA) resulting in frame-shifts (p.Cys28fs and p.Lys411fs, respectively) and PTCs. In one patient a 2-nt deletion (c.1894_1895delGA) was detected, and a 2-nt duplication (c.1910_1911dupTA) was observed in another patient. These two also lead to frame-shifts and PTCs (p.Glu632fs and p.Gln638fs, respectively). In addition, a 4-nt duplication

(c.1037_1040dupAGAT), also leading to a frame-shift (p.Met347fs) and PTC, was detected in one patient.

In one patient, no PCR products were amplified from exons 15 and 16, while other exons amplified normally. A long-range PCR from exon 14 to 17 produced a 900-bp product, while the genomic distance between these exons is over 9 kb. Sequencing of this PCR product revealed a genomic deletion of 8603 bp (*IVS14+506_IVS16-206del*). This genomic deletion was also detected in Southern analysis using several different restriction enzymes. On the protein level this mutation leads to a frame-shift (p.Arg439fs) and PTC. The deletion breakpoints in introns 14 and 16 are embedded within identical *AluY* repeat sequences, suggesting *Alu*-mediated homologous recombination as a deletion mechanism.

In addition to the above ten mutations producing PTCs, two single nucleotide substitutions, c.326G>C and c.965G>T, resulting in missense mutations in exons 5 and 12 (p.Cys109Ser and p.Gly322Val) were seen in the patients (see section 2.1 for further results and discussion). Both of these amino acids, the cysteine at position 109 and the glycine at position 322, are evolutionarily conserved down to zebrafish.

Additionally, a homozygous missense change (c.398C>T; p.Ala133Val) was seen in a Turkish patient homozygous for the p.Glu632fs mutation. This change was seen in heterozygous form in the parents of the patient, and was not present in the healthy brother of the patient (who did not carry the p.Glu632fs mutation), indicating that both changes reside in the same chromosome in both parents. This change was not seen in 100 Turkish control chromosomes, nor was it present in 100 CEPH control chromosomes. Further, the alanine at position 133 is evolutionarily conserved. However, when analysed with the PolyPhen program (Polymorphism Phenotyping; <http://genetics.bwh.harvard.edu/pph>), the p.Cys109Ser and p.Gly322Val mutations are predicted to be damaging while the p.Ala133Val change is predicted to be benign. Whether this is a pathogenic mutation or a rare normal polymorphic variant cannot be determined with the present data. However, the p.Glu632fs change is most likely the disease associated change in this family since it is likely to trigger NMD and lead to degradation of mRNA. Thus no p.Ala133Val mutant protein is likely to be produced.

The fourteenth change we detected was a splice site mutation (c.860G>A), affecting the 5' splice acceptor site of exon 10. In mRNA this results in skipping of

exon 10 and leads to an in-frame deletion of 17 amino-acids from the TRAF domain (p.Glu271_Ser287del).

Altogether 190 CEPH control alleles were analysed for each novel mutation. While the p.Gln638fs mutation was observed in heterozygous form in one control individual, the other mutations were not seen among the control alleles.

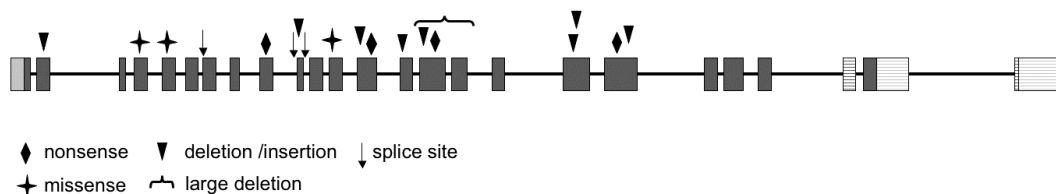
In some patients, no mutations were found in the exonic sequences, flanking intronic sequences or in the putative 600-bp promoter region. We looked for large genomic rearrangements using Southern analysis, but no differences were detected between these patients and controls. As no RNA was available from these patients, mutations in introns or regulatory regions affecting splicing or the transcript levels may well have remained undetected. Moreover, even though the 8.6-kb genomic deletion seen in homozygous form in one patient was detectable in Southern analysis, small genomic rearrangements, especially if in heterozygous form, may have been undetected. The existence of such rearrangements are likely, due to the high number of *Alu*-repeat sequences in introns of *TRIM37* (approximately one repeat every kb), as other genes with high numbers of *Alu* repeats have high frequencies of pathogenic deletions involving *Alu* repeats (Rüdiger et al., 1991; Bertolini et al., 1992). Additionally, the possibility of genetic locus heterogeneity in mulibrey nanism cannot be ruled out.

With six mutations reported previously (Avela et al., 2000; Jagiello et al., 2003; Kallijärvi et al., 2005), the total number of mulibrey nanism-associated mutations in *TRIM37* is now 19 (Table 9). The p.Ala133Val change is not included in this number, as it may be a rare polymorphism with no effect on *TRIM37* function. No mutational hotspots are seen and the 19 *TRIM37* mutations are distributed evenly from exon 2 to exon 19 (Figure 7). Apart from the Finnish founder *TRIM37* mutation, accounting for 98% of disease associated chromosomes in Finnish patients (Avela et al., 2000), all other mulibrey nanism-associated mutations were observed in only one or two families.

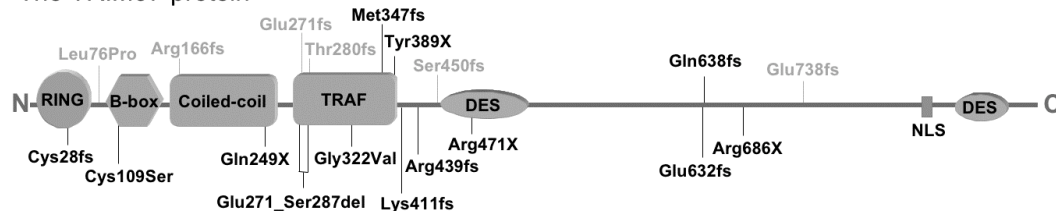
Table 9. Mutations in *TRIM37* associated with mulibrey nanism.

The FinMajor mutation, present in 98% of Finnish disease associated chromosomes and all Finnish patients, is shown in bold. All other mutations are seen in only one or two families.

Mutation	Type	Exon	Predicted consequence on protein product	Reference
c.81delG	deletion	2	p.Cys28fs; NMD	unpublished
c.227T>C	missense	4	p.Leu76Pro	Kallijärvi et al. 2005
c.326G>C	missense	5	p.Cys109Ser	II
c.493-2A>G	splice-site	7	p.Arg166fs; NMD	Avela et al. 2000
c.745C>T	nonsense	9	p.Gln249X; NMD	I
c.810-1G>A	splice-site	10	p.Glu271fs; NMD	Jagiello et al. 2003
c.838_842delACTTT	deletion	10	p.Thr280fs; NMD	Avela et al. 2000
c.860G>A	splice-site	10	p. Glu271_Ser287del	II
c.965G>T	missense	12	p.Gly322Val	I
c.1037_1040dupAGAT	duplication	13	p.Met347fs; NMD	I
c.1166A>G	nonsense	13	p.Tyr389X; NMD	unpublished
c.1233delA	deletion	14	p.Lys411fs; NMD	unpublished
c.1313+507_1668-207del	large deletion	15,16	p.Arg439fs; NMD	I
c.1346dupA	duplication	15	p.Ser450fs; NMD	Avela et al. 2000
c.1411C>T	nonsense	15	p.Arg471X; NMD	I
c.1894_1895delGA	deletion	18	p.Glu632fs; NMD	unpublished
c.1910_1911dupTA	duplication	18	p.Gln638fs; NMD	unpublished
c.2056C>T	nonsense	19	p.Arg686X; NMD	I
c.2212delG	deletion	19	p.Glu738fs; NMD	Avela et al. 2000

The *TRIM37* gene

The TRIM37 protein

**Figure 7. Schematic representation of the *TRIM37* gene and protein showing the positions of the 19 mutations reported to date in mulibrey nanism patients.**

In the gene picture the coding regions are in dark grey and untranslated regions in light grey. The regions that are alternatively present in the major alternative splice variants are striped. In the protein picture mutations described in this study are in black and mutations reported previously in grey letters.

Fifteen of the 19 *TRIM37* mutations result in PTCs predicting truncated protein products, or presumably destining the mRNAs to degradation by NMD (Mendell and Dietz, 2001). *TRIM37* mRNA expression in lymphoblastoid cells of Finnish patients with the Finnish major mutation was studied by quantitative PCR and only one third of the expression of control individuals was detected. This suggests that at least the Finnish major mutation leads to down-regulation of *TRIM37* mRNA.

2.1 Functional studies of the mutations predicting protein products (I, II)

Since most of the *TRIM37* mutations underlying mulibrey nanism predict PTCs and presumably do not produce protein, the functional consequences of the two missense mutations (p.Cys109Ser, p.Gly322Val) and the p.Glu271_Ser287del mutation were studied further. The cysteine to serine change at position 109 resides in the B-box of the TRIM domain (Figure 7). It affects a conserved cysteine residue in a flexible loop region of the B-box (Borden et al., 1995) and may interfere with the zinc binding properties of the B-box. The glycine to valine substitution at position 322 is located in the TRAF domain of *TRIM37* (Figure 7). Glycine is the smallest amino acid and is often located in critical positions for correct protein folding. The substitution of glycine with the bulkier valine may thus result in misfolding of the protein. The skipping of exon 10 due to the splice site mutation leads to deletion of the 17 first amino acids from the TRAF domain (Figure 7).

2.1.1 Subcellular localizations of the mutant proteins (I, II)

The subcellular localizations of the p.Cys109Ser, p.Gly322Val and p.Glu271_Ser287del mutant proteins were studied in transiently transfected BHK and COS-1 cells. In contrast to the granular pattern seen with the wild-type *TRIM37* (Kallijärvi et al., 2002), the mutant proteins showed homogenous cellular staining, indicating that the mutant proteins lose the peroxisomal localization (Figure 8). None of the mutant proteins formed aggresomes, which have previously been reported for the ectopically expressed wild type *TRIM37* (Kallijärvi et al., 2005).

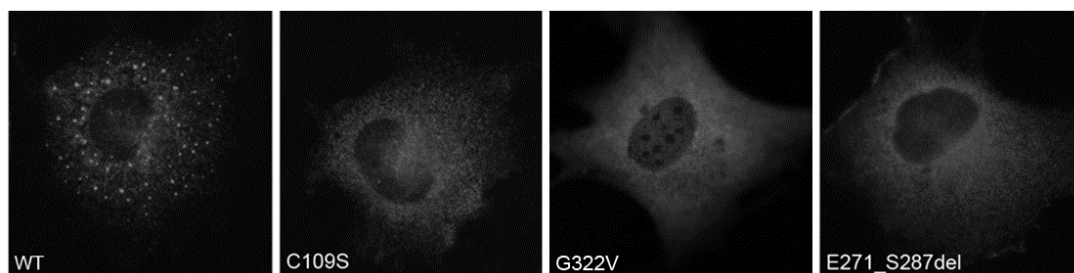


Figure 8. Subcellular localizations of the wild-type and mutant TRIM37 proteins.

COS-1 cells transiently transfected with HA-tagged wild-type or mutant TRIM37 and analysed by anti-HA antibody. While the wild type TRIM37 shows granular cytoplasmic localization all three mutant proteins show homogeneous cytoplasmic staining, indicating loss of peroxisomal localization.

Two of these mutations affect the TRAF domain and one the B-box domain of TRIM37, thus the cytoplasmic localizations were somewhat surprising since the coiled-coil region, which has previously been considered to be necessary and sufficient for proper subcellular targeting of TRIM family proteins (Reymond et al., 2001), is intact in all these mutant proteins. Thus our data suggests that even though the coiled-coil region is necessary for the proper subcellular localization of TRIM37 it is not sufficient in itself, as the B-box and TRAF domains also play roles in targeting TRIM37 in the cell. However, since TRIM37 has no peroxisomal targeting signals, the localization is most likely dependent on interaction with another, yet unidentified, protein and both the TRAF and the B-box domains have been implicated to have a role in interactions (Borden, 1998; Zapata et al., 2001). Thus, the mutations may interfere with TRIM37 interaction with the protein responsible for the peroxisomal localization. Whether the mutations directly affect the interacting domains of TRIM37 or merely change the overall folding of the protein and thus indirectly affect the interaction properties of TRIM37 is not known. Whatever the mechanism by which the mutant proteins lose the peroxisomal localization, the false subcellular localization suggests non-functionality of these mutant proteins. It also implies that peroxisomal localization is vital for the proper function of TRIM37.

2.1.2 Ubiquitin ligase activities of the mutant proteins (II)

As TRIM37 has been shown to possess ubiquitin E3 ligase activity, and a p.Leu76Pro mutation in the TRIM domain has been shown to result in diminished autoubiquitination of the mutant TRIM37 (Kallijärvi et al., 2005), the effect of the

p.Cys109Ser B-box mutation affecting the TRIM domain on the ligase activity was studied in cultured cells by studying autoubiquitination of the mutant protein. Expression constructs of full-length TRIM37 or its TRIM domain and hexahistidine-ubiquitin expression plasmid were co-transfected into COS-1 cells and immunoprecipitated by aminoterminal HA-tag to enrich the proteins. The immunoprecipitates were analysed by immunoblotting with either anti-ubiquitin or anti-HA antibodies. A high molecular mass ubiquitin positive smear, similar to the one seen with the wild type protein, was seen for the mutant protein suggesting that it has retained its E3 ligase activity (Publication II; Figure 3a). Similar results were achieved in *in vitro* ubiquitination assays, where GST-TRIM fusion proteins were incubated with ubiquitin in rabbit reticulocyte lysates (Publication II; Figure 3b). These results of a B-box mutation not affecting the ubiquitin ligase activity of TRIM37 are in line with previous studies where the RING finger has been shown to be essential for ubiquitin E3 ligase activity in RING proteins in general as well as in TRIM37 (Pickart, 2001a; Kallijärvi et al., 2005).

The two other new mutations, p.Gly322Val and p.Glu271_Ser287del, affect the TRAF domain of TRIM37 and thus are not likely to affect the E3 ligase activity. However, the autoubiquitination properties of the p.Glu271_Ser287del mutation were analysed in co-immunoprecipitation experiments with ubiquitin and the mutant TRIM37 protein. As expected, no difference could be detected between the wild-type TRIM37 and the deletion mutant. Further, the E3 ligase activity of the p.Gly322Val mutant protein has been studied outside of this study and similar autoubiquitination activity to wild-type TRIM37 has been detected for the mutant protein (Kallijärvi et al., 2005). Thus, none of these three mutations seem to affect the ubiquitin ligase activity of TRIM37.

2.1.3 Interaction properties of the p.Cys109Ser mutant (unpublished)

The B-box motif has been reported to participate in interactions of TRIM proteins (Borden, 1998). For example, a B-box missense mutation in TRIM18 that underlies OS has been shown to reduce the affinity of the B-box to its interaction partner $\alpha 4$, and thus lead to OS (Schweiger and Schneider, 2003), suggesting that B-boxes are critical for the correct interaction properties of TRIM proteins. Thus, as the loss of ubiquitin E3 ligase activity is not responsible for the loss-of-function of p.Cys109Ser

mutant TRIM37, the interaction properties of the mutant protein were studied. GST-fusion proteins of the mutant and wild-type TRIM37 were produced, and their binding to three putative interaction partners, SULT1A2 (sulfotransferase 1A2), NICE-3 and prohibitin, identified in yeast-two-hybrid screens (Kallijärvi, 2006), were studied with GST pull-down assays. The mutant protein bound all three putative interaction proteins, but the binding was weaker than that seen with the wild type protein (Figure 8), implying that the mutation interferes with the interaction properties of TRIM37. These data are very preliminary and whether the putative interaction partners also bind TRIM37 *in vivo* is unknown.

Impaired interaction properties may also underlie the pathogenesis of the p.Gly322Val and p.Glu271_Ser287del mutations, as they affect the TRAF domain and TRIM37 has been shown to bind to other TRAF proteins and itself via the TRAF domain (Zapata et al., 2001).

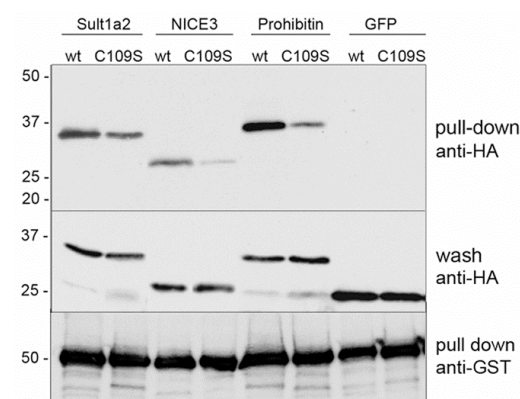


Figure 9. GST- pull down assays of the TRIM domain of TRIM37 and its putative interaction partners.

Putative interaction partners of TRIM37 were translated *in vitro* and the reaction products were incubated with either wild type or p.Cys109Ser mutant TRIM-GST fusion protein coupled to glutathione-sepharose beads. The bound proteins were analysed by immunoblotting with an anti-HA antibody. The mutant protein binds all three interaction partners, but the binding is weaker than for the wild type protein (upper panel). Green fluorescence protein (GFP) was used as a negative control that does not interact with TRIM. All *in vitro* translated proteins were detected in approximately equal amounts in the first wash after pull down (middle panel) and the GST-TRIM fusion protein was detected in approximately equal amounts with anti-GST antibody (lower panel).

2.2 Genotype-phenotype correlation

In recessive disorders the disease is usually due to the loss of functional protein and all changes causing loss-of-function result in a similar phenotype. In dominant

disorders, however, mutations often cause gain-of-function, i.e. the mutated protein gains an abnormal function, and thus different mutations may result in differences in phenotypes or even completely different diseases.

The 19 *TRIM37* mutations identified to date all seem to produce loss-of-function alleles, and consequently no genotype-phenotype correlation is seen in mulibrey nanism patients. However, the phenotypic picture of mulibrey nanism patients is variable. The overall appearance is very similar between patients but several different features can be seen, only some of them present in all patients, and while some patients can live a nearly normal life others have severe health problems. This variability is also seen among the Finnish patients homozygous for the Finnish founder mutation (Perheentupa et al., 1970; Perheentupa et al., 1973; Karlberg et al., 2004a), implying that the variability is not directly due to the *TRIM37* dysfunction but rather to other modifying genetic or environmental factors. All patients show the key clinical characteristics of prenatal-onset growth failure, dysmorphic craniofacial features, J-shaped sella turcica, hepatomegaly and moderate or severe signs of mulibrey heart disease (Perheentupa et al., 1970; Perheentupa et al., 1973; Karlberg et al., 2004a). Other organ manifestations such as hypothyroidism, hypoplasia of endocrine organs, ovarian fibrothecomas, liver tumors and Wilms' tumors are variably present in some patients. Thus, it looks like *TRIM37* mutations cause a variable, yet distinct clinical phenotype with the variance in the phenotype being caused by modifying genetic or environmental factors.

The clinical picture of mulibrey nanism resembles other growth disorders, and as it is extremely rare outside of Finland, most clinicians may have never encountered a mulibrey nanism patient. Thus, several mulibrey nanism patients without a proper diagnosis may exist around the world. Also, as we could not detect mutations in all the patients studied, a probable reason for this is that they may not suffer from mulibrey nanism but instead have some other growth restriction syndrome with similar features. During this study approximately 30 Finnish and 15 non-Finnish patients with short stature and some mulibrey-like features, but not a phenotype fulfilling the diagnostic criteria formed on the basis of clinically relatively uniform Finnish patients molecularly confirmed to have *TRIM37* mutations, were screened for *TRIM37* mutations, but no changes were detected in them.

3. Mouse Trim37 (IV, unpublished)

3.1 Comparative genomics

Comparison of the *Trim37* genes from different species revealed high evolutionary conservation among all vertebrates, whereas the *Trim37* gene is not seen in the genomes of lower species. In all vertebrates studied (human, chimp, mouse, rat, zebrafish) the cDNA sequences showed high homology and the genomic structures of the orthologs were identical with exon-intron junctions at identical positions.

Comparison of Trim37 proteins reveals even higher evolutionary conservation and homology than that seen on the gene level. Especially the N-terminal part of the protein that includes the TRIM domain as well as most of the TRAF domain (aa 1-385) is highly conserved. The N-terminal part of human TRIM37 shows 100% identity with other mammalian, 99% identity to the chicken and 96% identity to the zebrafish Trim37 proteins. Though the C-terminal part of the protein is much less conserved than the N-terminus, high conservation is seen for the whole protein, with the mouse Trim37 showing 92% identity to the human protein.

Figure 10 shows alignment of human, mouse, chicken and zebrafish Trim37 proteins.

3.2 Characterization of the mouse *Trim37* gene (IV)

The pathogenetic mechanisms behind a developmental disorder like mulibrey nanism, are best studied in an animal model. For the future generation of *Trim37* knock-out mice, the mouse *Trim37* gene (NM_197987) was characterized in detail.

As expected from the high homology at the protein level, the mouse gene is highly homologous to the human gene, with the cDNA sequences showing 88% identity between human and mouse.

Alignment of the mouse *Trim37* cDNA sequence to mouse genomic sequence revealed identical genomic structure with the human gene. The exon-intron boundaries, at identical positions with the human gene, and the sizes of the exons and introns are presented in Table 10.

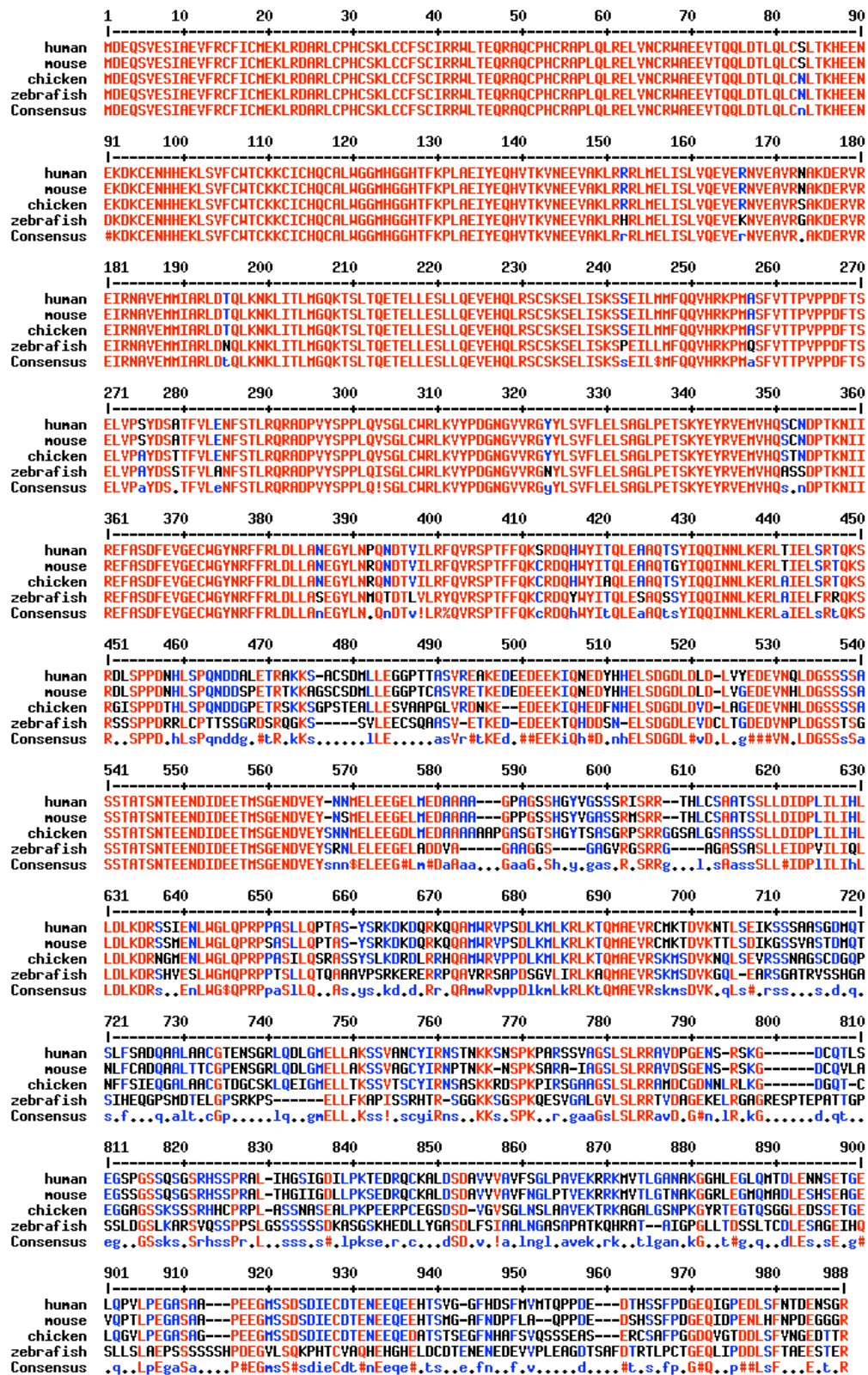


Figure 10. Alignment of human, mouse, chicken and zebrafish TRIM37 proteins.

High evolutionary conservation is seen among all species. Especially the amino-terminal part of the protein with the functional TRIM (aa 5-254 for human TRIM37) and TRAF (aa 271-405 for human TRIM37) domains show high conservation.

Table 10. Exon-intron boundaries of mouse *Trim37*.

Exon nr	Exon size (bp)	3' Splice Acceptor Site	5' Splice Donor Site	Intron nr	Intron size (kb)
1	418		GAGTGTGGAGgtgaggggca	1	2.2
2	102	ttgttttcagAGCATTGCTG	CTGTATTAGGgtaagttgta	2	7.7
3	41	gcctccttagCGCTGGCTGA	CTCACTGTCGgtaagtgttt	3	2.9
4	117	ttcttttcagTGCTCCACTC	AGAAAGACAAGtgcgtagtt	4	2.5
5	88	taatgaatagATGTGAAAAT	GGGAGGAATGgtgagcagaa	5	2.2
6	123	atttctgtagCATGGTGGAC	TCAAGAAGTGgtaagattac	6	1.3
7	124	tatcttctagGAAAGAAATG	ACTCTCATGGgtgagtgtgc	7	2.2
8	68	ttttaaacagGTCAGAAGAC	AGAACATCAGgtgataaat	8	10.5
9	125	cctcacacagTTGCGGTCTT	ACTTTACCAGgtaacagttt	9	3.9
10	51	ttcttttagTGAATTGGTA	AGAACTTCAGgtaagaggtt	10	2.7
11	82	tttctgtagCACTTTGCGG	AGTTTACCCAgtaagtttt	11	0.8
12	77	ttttttaagGATGGAAATG	AAACTTCCAAgtaagtgtt	12	10.3
13	180	tgcattttagGTATGAATAT	TGATTTTAAGgtagtttgag	13	4.4
14	115	attctttaagGTTTCAGGTG	TCTTAAAGAGgtaaggcaat	14	2.3
15	219	attttaaaagAGACTGACTA	AGACTATCATgtaataagag	15	1.3
16	137	ttcctttagCATGAGCTCT	AGGAGACCATgtgagtcacca	16	3.6
17	86	ctatttgcagGTCTGGGGAA	GGTCCTCCAGgtacagaggc	17	6.6
18	195	tcttttgcagGTAGTAGCCA	CAGCCCACAGgtaaaataat	18	4.3
19	309	ctttttgaagCATCATATTC	ATACGAAACCgtaagtgcag	19	4.3
20	123	tatttttcagCCACAAATAA	CTGGCTGAAGgtaaaagctga	20	0.7
21	190	ttcctgaagGCTCCTCGGG	TCACCTTGGGgtaaaataata	21	2.8
22	119	cttctttgagGACTAATGCA	CCTGAGGAAGgtgagcattg	22	6.6
23	111	ttcctgccagGAATGAGTAG	GAGGTGGAAGgtaattgccca	23	1.7
24	79/>2431*	tttttcttagATTACATTC	GAGGTGGAAGgtaattgccca	24	32.3
25	>281	ccctctctagACTGCTTGTT			

*Two forms of exon 24 representing alternative splice variants are presented

The sequences of the 5' flanking regions of the human and mouse *Trim37* genes also show high homology (Publication III; Figure 2). Several common putative transcription factor binding sites are present in human and mouse sequences and basal promoter activity is predicted to be present in this region in both species.

3.2.1 Alternative splice variants (IV)

As human *TRIM37* shows a wide range of alternative splice variants, we wanted to see whether the same was true also for the mouse gene. Northern analysis with several

different probes from the *Trim37* coding region revealed three alternative mouse transcripts of 3.5, 4.5 and 5.5 kb (Publication IV; Figure 1), whereas the human *TRIM37* gene shows only two alternative variants in Northern analysis (Publication III; Figure 4). To characterize these three transcripts and to find out whether they represented the same alternative variants we saw in the human transcriptome (section 1.2), *in silico* analysis of database sequences and RT-PCR experiments on mouse mRNA were performed.

A blast search of mouse ESTs returned over 300 mouse *Trim37* sequences, representing eight alternative variants (listed in Table 11). As the human gene has a variant with an additional 3' exon, we looked especially for a similar transcript in mouse. Interestingly, mouse also showed a transcript with 25 exons (*Trim37b*), with the splice site at the identical position as in the human gene. However, while in humans the two major transcripts encode identical protein products of 964 amino acids, mouse *Trim37b* has 47 extra C-terminal amino acids, producing a 1008 aa protein, while mouse *Trim37a* produces a 961 aa polypeptide. Among the transcripts representing *Trim37a* (24 exons), several ESTs showed utilization of two alternative polyadenylation signals with polyA tracks 1145 and 2348 nucleotides downstream of the termination codon. RT-PCR and sequencing confirmed these alternative 3' sequences for mouse *Trim37*.

Table 11. Alternative mouse *Trim37* transcripts from database ESTs

Nucleotide numbering starts with +1 at the A of the ATG translation initiation site (nucleotide 398 in NM_197987)

EST clone(s)	Difference to NM_197987	Predicted protein product
XM_109795 BU583805	<i>Trim37b</i>	1008 aa TRIM and TRAF intact
CN533816 CN676751	del part of exon 8 (c.673_684)	957 aa TRIM and TRAF intact
CD807942	del exon 20	920 aa TRIM and TRAF intact
CF749419	del exon 19	858 aa TRIM and TRAF intact
AB093271	del part of exon 9 (c.685_698)	228 aa PTC*
CF726590	del exons 7-13, parts of exons 6 and 14 (c.418_1222)	164 aa PTC
CB519726	del exon 3	62 aa PTC

*PTC = premature termination codon

Based on the EST sequences and human transcripts we hypothesized that the three transcripts seen in northern analysis could be explained by different 3' sequences; as the coding region and the 5' UTR together sum up to 3.3 kb, *Trim37a* with two

different polyA signals could account for the 4.5-kb and 5.5-kb transcripts and *Trim37b* for the 3.5-kb transcript. This was proven to be true, as a *Trim37a* 3' UTR specific probe detected the 4.5 and 5.5-kb transcripts, with high expression for the 4.5-kb transcript in testis and the 5.5 kb-transcript in brain, and only the 3.5-kb transcript, with high expression in testis, was seen when a *Trim37b* specific probe was used (publication IV; Figure 3). Apart from these three major variants, six other alternative transcripts were detected in mouse. The exon 23 deletion seen in human splice variants was not among them. Nor was the del23 variant seen in RT-PCR experiments on mouse tissues, whereas it was readily amplified from human mRNA. As in humans, most of the alternative splice variants seem to be expressed at low levels in the mouse, and the same variants could not be detected in humans. Alternative variants were also searched from rat databases, and the only variants seen in the rat were the *Trim37a* and *Trim37b* transcripts, with rat *Trim37b* producing a similar longer protein product as mouse *Trim37b*. These results further suggest that the variants alternating in their 3' sequences are the major functional alternative transcripts of *Trim37*. Alternative splicing seems to have a regulative role in controlling the expression of *Trim37*, and the other transcripts are likely to be produced for this purpose.

3.3 Expression of Trim37 in mouse tissues (IV)

The detailed expression pattern and distribution of *Trim37* expression in embryonic and adult mouse tissues was further studied by immunohistochemical methods. Antigen affinity-purified fractions of the anti-human TRIM37 antisera (Kallijärvi et al., 2002) were used for the detection of *Trim37*.

3.3.1 Expression of Trim37 in embryonic tissues (IV)

The distribution of *Trim37* expression in embryonic tissues, showed a highly similar pattern to the previously reported mRNA expression (Lehesjoki et al., 2001). A weak staining was seen already at the earliest stage analysed (E9) and at later stages (E10 and E12.5) a stronger staining became apparent in many tissues. The staining was highest in heart, somites, developing lung and foregut. Strong staining was also detected in the surface ectoderm of the embryo. At E15.5 strong staining was seen in

the epithelia of intestine and kidney. Also, the developing pancreatic buds, the surface ectoderm and the dorsal root ganglia stained intensively.

3.3.2 Expression of Trim37 in adult tissues (IV)

Adult mouse tissues showed a strikingly restricted and tissue-specific expression of Trim37. All tissues of the mulibrey acronym (muscle-liver-brain-eye) stained positively (Figure 11; uppermost panel). Relatively weak, fiber-specific distribution of Trim37 was seen in a subset of muscle fibers of both skeletal and heart muscle, while the ganglia that innervate the muscle tissue showed strong expression. As mild hypotonia is the only muscle related manifestation in mulibrey nanism patients, it is plausible that, as suggested previously (Karlberg et al., 2004a), the muscle hypotonia is related to mild neurological dysfunction. In the liver, weak nuclear staining of Trim37 was seen in hepatocytes.

Strong expression of Trim37 was observed in neuronal tissues. Many regions of the brain showed staining in restricted neuronal populations. Mulibrey nanism patients have normal intelligence, but mild structural defects are present in the central nervous system (CNS) and the patients manifest mild neurological defects, including mild delay in motor and speech development and mild muscle hypotonia (Karlberg et al., 2004a). Overall, the immunohistochemical findings in the CNS would suggest a stronger neurological defect than what is seen in the patients. Yellowish dots in the retinal mid peripheral region are one of the major diagnostic criteria for mulibrey nanism (Karlberg et al., 2004a). However, despite the intense photoreceptor specific expression of Trim37 seen in the retina, the vision is not affected in mulibrey nanism patients.

Strong Trim37 expression was detected in many tissues of the digestive system, including the mucinous tissue of salivary glands, mucus-producing goblet cells of the intestine and the enteric ganglia (Figure 11; middle panel). Interestingly, many mulibrey nanism patients suffer from poor feeding during infancy (Karlberg et al., 2004a). In the light of the expression pattern of Trim37 in the digestive system, it would be tempting to speculate that the feeding difficulties could be related to enteric ganglia regulated functions i.e. bowel movements or mucus secretion by goblet cells.

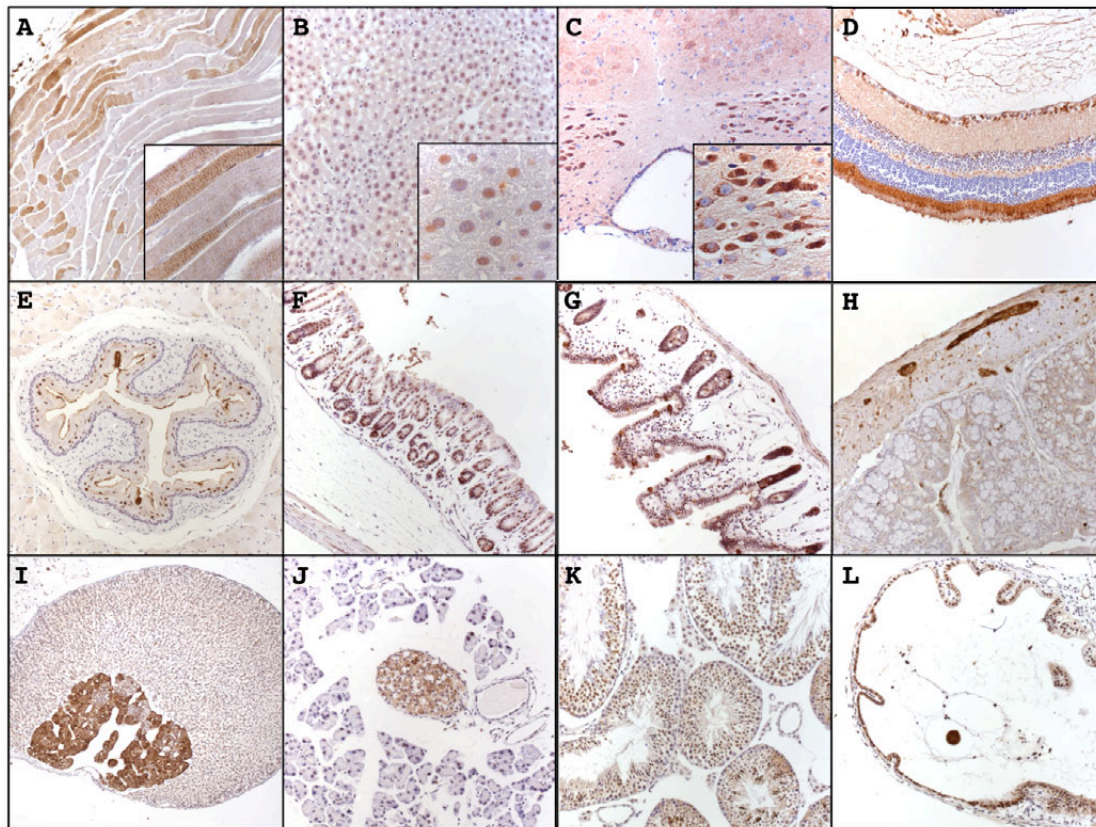


Figure 11. Immunohistochemical analysis of Trim37 distribution in mouse tissues.

All tissues of the mulibrey acronym stained positively for Trim37. Skeletal muscle (A) shows fiber-specific staining, in the liver (B) weak nuclear staining is seen in the hepatocytes, in the brain stem (C) a subset of neurons stain strongly and strong staining is seen in the photoreceptor layer of the retina (D). Many tissues of the digestive system show high expression of Trim37. The epithelia of the oesophagus (E) and stomach (F) stain positively, in the ileum (G) goblet cells stain strongly and in the colon (H) the enteric ganglia stain strongly. Some endocrine tissues also stain positively for Trim37. The adrenal medulla (I) shows very intense staining and a distinct staining is seen in the islets of Langerhans in the pancreas (J). In the gonads strong staining for Trim37 is seen in the germ cells. In testis (K) developing sperm stain strongly and in the ovary (L) a subset of maturing oocytes stain strongly. Magnification is 200X and 1000X in the insets.

Several endocrine organs showed intense staining of Trim37 (Figure 11; lowermost panel). Strong expression was evident in a subset of cells of adenohypophysis, the endocrine part of the pituitary gland, and adrenal medulla. Weak staining was also present in the endocrine component of the pancreas, i.e. the islets of Langerhans.

In post-pubertal mice, intense staining for Trim37 was seen in germ cells (Figure 11; lowermost panel). In testis, the developing sperm showed strong stage-specific staining from type B spermatogonia to early round spermatids. In ovaries strong staining of oocytes was seen, and also the granulosa cells, luteal gland and the epithelium of the fallopian tube stained positively. Female mulibrey nanism patients present incomplete sexual maturation and develop premature ovarian failure

(Karlberg et al., 2004b). In male patients, testicular hypoplasia and reduced number of sperm is encountered (Lipsanen-Nyman, 1986). Thus, the immunohistochemical findings in mouse gonads are in agreement with the clinical findings of mulibrey nanism patients.

Taken together, these data suggest a role for Trim37 in many neural crest-derived tissues, as well as in a subset of endocrine tissues and the gonads. The expression of Trim37 in mouse tissues is in agreement with the clinical findings in human patients. This data, together with the characterization of the mouse Trim37 gene, provides the basis for the development and analysis of Trim37^{-/-} mice.

CONCLUSIONS AND FUTURE PROSPECTS

In this study the *TRIM37* gene was characterized in detail and mutations underlying mulibrey nanism were identified and characterized. In general, this thesis work forms the basis for the molecular diagnostics of mulibrey nanism as well as for the study of the molecular pathogenesis behind this disorder.

Clinical diagnosis of mulibrey nanism can sometimes be difficult and molecular genetic confirmation can be offered to confirm the diagnosis. Most of the Finnish mulibrey nanism patients are homozygous for the Finnish major mutation while the three minor mutations, identified in four Finnish patients, are seen only in compound heterozygous form with the major mutation. Thus, for molecular diagnostics in Finland, screening for the major mutation seems to be sufficient and other mutations should be looked for only in clinically affected patients heterozygous for the major mutation. Elsewhere in the world, private sporadic mutations underlie mulibrey nanism and for diagnosis of non-Finnish patients the whole *TRIM37* gene should be sequenced.

In this study, several patients with short stature and some mulibrey-like features, but who did not fulfill the diagnostic criteria for mulibrey nanism, were screened for *TRIM37* mutations, but no changes could be detected. Thus, *TRIM37* mutations seem to be associated only with the distinct clinical phenotype of mulibrey nanism (Karlberg et al., 2004a).

Most of the *TRIM37* mutations underlying mulibrey nanism result in PTCs and thus are likely to trigger NMD and lead to degradation of mRNA. Consequently, little or no protein is produced and the mutations act as loss-of-function alleles. Since no genotype-phenotype correlation is seen in mulibrey nanism patients, all mutations seem to produce loss-of-function alleles. Thus, the four *TRIM37* mutations that are likely to express mutant proteins may turn out to be important in assessing the function of TRIM37, as they affect important functional domains of TRIM37. These mutations either alter the subcellular localization of TRIM37, possibly by affecting the interaction properties of TRIM37, or decrease the ubiquitin ligase activity of TRIM37, suggesting that both the ligase activity and the peroxisomal localization are needed for the proper function of TRIM37.

The normal physiological function of TRIM37 is as yet unknown. However, since the ubiquitin E3 ligase activity suggests a role for TRIM37 in proteasomal protein degradation, the loss-of TRIM37 function may lead to the accumulation of its yet unidentified substrates. The identification of these substrates would be valuable as it could elucidate the pathogenesis behind mulibrey nanism.

Expression of TRIM37 seems to be controlled at several levels. The promoter region is typical for a house-keeping gene and *TRIM37* mRNA is present in all tissues but the level of expression varies between different tissues and a specific splice variant is present at least in testis. The existence of several seemingly non-functional splice variants implies that TRIM37 expression is regulated translationally. This idea is further supported by the strictly cell specific protein expression pattern of TRIM37. The cell specific distribution of TRIM37 expression also suggests cell specific functions for TRIM37. Characterization of this expression pattern in mouse tissues may give clues for dissecting the clinical phenotype of mulibrey nanism as well as for the pathogenetic mechanisms behind the disorder.

Despite the current information on the TRIM37 gene and protein, the biochemical pathways behind mulibrey nanism are still unknown and many important questions remain to be answered. The generation of *Trim37*^{-/-} mice, which is currently underway, opens up new possibilities for the study of Trim37 and mulibrey nanism. The high homology between human and mouse Trim37 makes the mouse a promising candidate organism to model mulibrey nanism, but whether the mice will replicate the human phenotype remains to be seen. However, the knock-out mice will provide an unlimited repository of affected tissues and cells, as well as an opportunity to study the pathogenetic mechanisms at the level of a whole organism. Mulibrey nanism affects several different tissues and developmental processes, growth and cellular metabolism. Thus, the *Trim37*^{-/-} mice may also provide ways to study development and growth in general as well as serve as a model for diabetes and metabolic syndrome.

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A handwritten signature in black ink, appearing to read 'Riihela', followed by a long horizontal flourish.

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